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13. ABSTRACT (Maximum 200 Words)

The Concept Award focused on investigating the expression on the biomarker PCDGF/GP88 in breast cancer and its effect on the acquisition of estrogen independence and tamoxifen resistance, a hallmark of breast cancer with poor prognosis. Specific aims set for this application were achieved. PCDGF expression was examined by immunohistochemistry in paraffin embedded breast cancer biopsies. A study with 206 samples showed that PCDGF was mostly negative in benign lesions. In contrast 67% of ductal carcinoma in situ (DCIS) and 80% of invasive ductal carcinoma (IDC) stained positive for PCDGF/GP88. In IDCs, PCDGF expression correlated with tumor grade, p53 and Ki67. In contrast, no correlation was found with ER/PR and Her-2 expression. Based on these results, we examined the effect of increased expression of PCDGF in ER+MCF-7 cell line as model system. We showed that increased of PCDGF expression in these cells led to estrogen independence and to resistance to the antiestrogen tamoxifen both in vitro and in vivo in mouse xenografts. Molecular studies demonstrated that PCDGF exerts its effect by blocking the apoptotic effect of tamoxifen. These data demonstrate the importance of PCDGF/GP88 as a novel target for the development of therapy for tamoxifen resistant breast cancer.

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Final Report for grant application DAMD17-01-1-0551

Introduction.

The purpose of this Concept Award was to investigate the expression of a novel biomarker called PC-Cell derived growth factor (PCDGF/GP88) in breast cancer biopsies. Our studies have established a solid foundation for the role for the novel autocrine growth factor PCDGF on the proliferation and tumorigenicity of human breast carcinomas.

PCDGF is an 88 kDa glycoprotein (also know as granulin precursor or progranulin) that was originally characterized in our laboratory). PCDGF was shown to act as an autocrine growth factor for breast cancer cells and to be overexpressed in human breast cancer cell lines in appositive correlation with the degree of tumorigenicity. WE showed hat inhibition of PCDGF expression by antisense PCDGF cDNA transfection led to a complete inhibition of tumor growth in mouse xenografts. In addition, our work established a novel mechanism for estrogen mediation of growth of mammary epithelial cells and provided the mechanism of the molecular events (overexpression of PCDGF and overexpression of PCDGF receptor) associated with transition from estrogen-dependence to estrogen-independence.
These data indicated that PCDGF was a critical player of breast cancer tumorigenesis (for review Serrero, 2003, provided in the appendix).

Based on these data, the present study supported by this grant application was devoted to investigate PCDGF expression in breast cancer biopsies to determine whether altered expression of PCDGF was observed in breast cancer biopsies and if PCDGF expression correlated with parameters of poor prognosis.

Body of the technical report.
In order to carry out these studies,
1) We first developed an anti-PCDGF antibody that could be used for immunohistochemistry (IHC). We have developed both polyclonal and monoclonal antibodies developed against human recombinant PCDGF that can be used for IHC application.

2) We demonstrated that these antibodies could be used for IHC studies in paraffin embedded breast cancer biopsies.

3) Based on this data, we have established a study of 206 biopsies obtained from two tumor repositories to examine PCDGF expression in benign lesions, ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC).
Studies were carried out to correlate PCDGF expression in these biopsies with clinical parameters and parameters of poor prognosis such as Ki67 index, p53 and Her2.

The data are summarized below and presented in detail in an article provided in the Appendix
Serrero, G and Ioffe O (2003) Expression of the novel autocrine growth factor PC-Cell Derived Growth Factor in human breast cancer tissue. Human Pathology. 34, 1148-1154.

PCDGF staining was observed in breast carcinoma whereas it was negative in benign breast epithelium. PCDGF expression was more common in invasive ductal carcinoma (80% cases positive) than in invasive lobular carcinoma (18% positive). No PCDGF staining was observed in lobular carcinoma in situ. Ductal carcinoma in situ expressed PCDGF in 66% of the cases.

Correlation of PCDGF expression with clinical parameters was carried out.
We show that PCDGF expression correlated strongly with nuclear grade in DCIS.
Similar correlation was observed between PCDGF expression and histologic grade of invasive ductal carcinoma.

Average Ki-67 index of PCDGF negative/weakly positive invasive carcinomas (30.3) was significantly lower than that of strongly PCDGF-positive tumors (48.8, $p<0.05$) indicating that PCDGF expression in IDCs correlated with proliferation index.

Positive correlation was also observed between PCDGF expression and p53 positivity. A larger percentage of tumors that expressed PCDGF with a staining intensity of 2+ or 3+ were p53-positive (44%) than PCDGF-negative tumors (25%), $p<0.05$.

In contrast, PCDGF expression was independent of c-erbB-2 overexpression and of ER/PR status. This result is interesting indicating that PCDGF is a novel therapeutic target that will address population distinct from the populations that have a Her2 overexpression.

This and the fact that PCDGF is expressed in 80% of IDCs would suggest that developing therapy based on PCDGF could address a population of breast cancer patients for whom targeted therapy is limited.

In conclusion, our study provides the first evidence of high incidence of PCDGF expression in human breast cancer in which it correlates with clinicopathological variables such as tumor grade, proliferation index and p53 expression. These characteristics as well as the absence of expression in benign breast tissue suggest an important role of PCDGF in breast cancer pathogenesis and make it a potential novel target for the treatment of breast cancer.

Future perspectives based on these data.
Based on these data and since we have found that high PCDGF expression was observed in ER^+ tumors as well as in ER^- tumors, we have postulated that PCDGF expression in ER positive tumors could play a role in modulating response to anti-estrogen therapy.
Based on this hypothesis, we have first carried out basic studies with ER positive breast cancer cell lines MCF-7 and shown that increased level of PCDGF in MCF-7 cells was associated with estrogen independence and tamoxifen resistance both in vitro and in vivo. These data were submitted in a manuscript that was accepted with revision for publication in Cancer Research.
Tangkeangsirisin, W., Hayashi, J., and Serrero, G (2003) PC-Cell Derived Growth Factor (PCDGF/progranulin) mediates tamoxifen resistance and promotes tumor growth of human breast cancer cells. Cancer Research. Accepted for publication.

It is then proposed to examine whether increased PCDGF expression in ER^+ IDCs correspond to patients that are non responsive or resistant to tamoxifen. Such study will be established at the University of Maryland where the PI is adjunct Professor and in Europe.

Key research accomplishments

1) Developed antibodies and experimental conditions for IHC studies of PCDGF expression
2) Obtained 206 paraffin embedded biopsies from two tumor repositories Univ. Maryland and Manitoba Breast Cancer Tumor bank.
3) Carry out pathological studies in 206 human breast cancer biopsies
4) Presentation of data at the DOD Era of Hope meeting in Orlando FA in 2002.
5) Preparation of a manuscript describing the pathological studies published in Human Pathology 2003.
6) Demonstrated that PCDGF expression in ER^+ breast cancer cells is associated with estrogen independence and tamoxifen resistance.
7) Developed a sandwich immunoassay to measure PCDGF level in frozen cytosols and in serum of breast cancer patients.
8) Develop clinical studies to examine therapy responsiveness and PCDGF level in breast cancer patients

Reportable outcomes

1) Training of graduate students

3 graduates students enrolled.
One graduated in June 2003. Presently a post-doc at Univ. California San Francisco
One will graduate in January 2004. Faculty in Thailand School of Pharmacy
One advanced to candidacy and will graduate in May 2005

2) Abstracts and publications supported by this application

Abstracts and invited presentations:

Ginette Serrero and Olga Ioffe (2002) Expression of PC-Cell Derived Growth Factor (PCDGF/progranulin) in breast cancer biopsies. DOD Era of Hope meeting in Orlando Florida. Poster presentation.

Wisit Tangkeangsirisin and Ginette Serrero (2003) Adipose Differentiation Related Protein (ADRP) induces differentiation of human breast cancer MCF-7 cells. Poster presentation, 2003 Annual Meeting of the American Association of Cancer Research. Washington DC.

Wes E. Kim and Ginette Serrero (2003) PC-Cell Derived Growth Factor confers Herceptin resistance to c-ErbB2 overexpressing human breast cancer cells. AACR Scholar-in Training Award and poster presentation, Annual Meeting of the American Association of Cancer Research. Washington DC.

Ginette Serrero, Wisit Tangkeangsirisin and Huifang Dai (2003) Role of PC-Cell Derived Growth Factor in breast cancer tumorigenesis. Invited conference Eurocancer 2003 meeting, Paris, France.

Ginette Serrero, Kate Tkaczuk, Nancy Tait and Huifang Dai (2003) Novel serum biomarker for monitoring breast cancer patients. San Antonio Breast cancer Symposium 2003. Poster presentation.

Ginette Serrero and Olga Ioffe (2003) Expression of PC-Cell Derived Growth Factor (PCDGF/progranulin) in breast cancer biopsies and correlation with prognostic markers. San Antonio Breast Cancer Symposium 2003. Poster presentation.

Publications:

Brown, M.R., Blanchette, J.O., Kuznetsov, V.A., Raffeld, M., Serrero, G., Bonner, R.F., Liotta, L.A., Emmert-Buck, M.R., Petricoin, E.F., Krizman, D.B., and Kohn, E.C. (2003) Differential Expression of the Granulin Epithelin Precursor gene in Epithelial ovarian Tumors identified through statistical analysis of microdissected cDNA libraries. Clin. Cancer Res. 9, 44-51.

Hoque, M, Tara M. Young, T.M., Chee-Gun Lee, C-G., Serrero, G., Mathews, M.B., and Pe'ery, T. The Growth Factor Granulin Interacts with Cyclin T1 and Modulates P-TEFb-Dependant Transcription. (2003) Mol. Cell. Biol. 23, 1688-702.

Wang, W, Kim, W, Hayashi, J and Serrero, G (2003) Expression and action of PCDGF in human Multiple Myeloma. Clin. Cancer Res. 9, 2221-2228. *

Serrero, G (2003) Autocrine growth factor revisited: PCDGF a critical player in breast cancer tumorigenesis. (review) Biochem Biophys Res Commun. 308, 409-413*

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Breakthroughs and Views

Autocrine growth factor revisited: PC-cell-derived growth factor (progranulin), a critical player in breast cancer tumorigenesis

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Abstract

PC-cell derived growth factor (PCDGF), also known as granulin precursor or progranulin, is the largest member of a family of growth modulators characterized by a unique cysteine-rich motif. Biological and pathological studies point out to the importance of this growth factor in breast cancer and other human cancers, where it stimulates proliferation and survival, and promotes metastasis. These studies suggest that PCDGF is a suitable therapeutic and diagnostic target for the development of novel cancer therapy and diagnosis.

Keywords: Breast cancer; PC-cell derived growth factor; Granulin/epithelin precursor; Progranulin; Tumorigenesis; Autocrine growth factor

Breast cancer is a major cause of morbidity and mortality in women worldwide. The progression from normal breast epithelium to metastatic breast cancer is a complex multistep process resulting from the uncoupling of the interactive systems controlling cell proliferation and differentiation, thus leading to extensive cellular growth [1]. Among the possible alterations in growth regulatory processes leading to neoplastic transformation, the one of interest for breast cancer has been the overexpression of specific growth factors in a cell already expressing their receptors, or the overexpression of growth factor receptor. It has been suggested that the escape from hormonal dependency, a hallmark of breast cancer recurrence when cells become estrogen-independent and refractory to hormone therapy was the result of the overexpression of autocrine growth factor(s) or receptors that may or may not be under the control of estrogen action [2]. Several growth factors have been shown to be overexpressed and to act as autocrine growth stimulators for breast cancer cells. Among them, particular attention has been given to the insulin-like growth factors (IGFs) and epidermal growth factor families [3]. In addition, breast cancer cells have been shown to overexpress growth factor receptors, such as erbB2 (Her2), member of the EGF receptor family [4]. In fact, inhibiting Her2 by Herceptin, the humanized anti-Her2 monoclonal antibody has provided a very powerful therapy for metastatic breast cancer. This has established the importance of targeting autocrine growth factor, growth factor receptors overexpressed in cancer cells to develop new and specific cancer therapies. This premise then emphasizes the importance of identifying growth factors and receptors overexpressed in human cancers that are required for their proliferation, survival or escape from apoptosis.

This review will provide information on the critical role of the autocrine growth factor, PC-cell derived growth factor (PCDGF), in breast cancer tumorigenesis. Experimental evidence will demonstrate that PCDGF is an effective target for the development of novel cancer therapy and diagnosis. Excellent reviews providing information about the structure, regulation, and biology of this family of growth factors are available for interested readers [5,6].

Characterization of PC-cell derived growth factor

PC-cell derived growth factor, also known as granulin precursor or progranulin, is an 88 kDa growth factor that

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Moreover, PCDGF staining was almost never observed in lobular carcinoma in situ, whereas 66% of ductal carcinoma in situ (DCIS) expressed PCDGF. PCDGF expression in DCIS correlated strongly with nuclear grade in DCIS and histological grades in IDC. Both ER positive and ER negative tumors had moderate to strong PCDGF expression. Positive correlation was found between PCDGF staining and Ki-67 proliferation index. Similarly, a larger percentage of tumors with moderate/strong PCDGF expression were p53 positive. In contrast, PCDGF expression was independent of c-erbB-2 overexpression. This study provides evidence of the high incidence of PCDGF expression in human breast cancer with positive correlation with clinico-pathological variables such as tumor grade, proliferation index, and p53 expression. These characteristics the absence of expression in benign breast tissue suggest an important role of PCDGF in breast cancer pathogenesis and make it a potential novel target for the treatment of breast cancer.

Overexpression of PCDGF/granulin precursor in other types of cancers

Increased expression of the 88 kDa glycoprotein has been reported in several other cancers including brain tumors, gliomas, ovarian cancer, renal carcinoma, and hematological cancers using a variety of experimental approaches. Progranulin was identified as one of 30 most up-regulated genes between acute myeloid leukemia cells with isolated trisomy 8 (AML + 8) and CD34+ cells purified from normal bone marrow [32]. It was one of the top 50 genes effective in discriminating between acute lymphoblast leukemia (ALL) and acute myeloid leukemia (AML) when measured by median vote relevance [33]. Differential immuno-absorption technique identified progranulin as a human glioma-associated growth factor gene [34]. Moreover, gene expression profiling reported the upregulation of progranulin in primary glioblastoma multiform tumors [35]. Immunohistochemical studies demonstrated that high-grade renal cell carcinoma presented high levels of progranulin expression compared to low-grade renal cell carcinoma and normal tissue [36]. Reverse transcription-PCR and immunohistochemistry were used to demonstrate increased PCDGF expression in invasive ovarian cancer cells. PCDGF level was high in invasive ovarian tumors, whereas it was undetectable in serous low malignant potential (LMP). Inhibition of PCDGF expression by antisense cDNA transfection in ovarian cancer cells led to reduction in cell growth, decrease in the S-phase fraction, and loss of density-independent growth potential [26]. Immunohistochemical studies in bone marrow smears of patients with multiple myeloma indicated that PCDGF expression was expressed in the myeloma cells and correlated with the presence of MM disease [27]. Using human MM cell lines as model system, it was shown that PCDGF mRNA and protein were expressed in several human MM cell lines and that treatment of the cells with anti-PCDGF neutralizing antibody caused inhibition of proliferation. These results demonstrated that, as with breast cancer cells, PCDGF/GP88 is an autocrine growth factor for MM cells and its expression correlates with disease status in pathological samples [27].

Conclusion: PCDGF/GP88 a critical factor in cancer tumorigenesis

Biological as well as pathological studies have demonstrated the critical importance of PCDGF/GP88 in several key steps of the tumorigenesis process. PCDGF/GP88 stimulates cell proliferation and survival, inhibits apoptosis, and stimulates cell migration and invasiveness. In breast cancer cells, increased expression of PCDGF has been associated with acquisition of resistance to anti-estrogen and chemotherapeutic agents. The studies carried out with breast cancer cells, ovarian cancer, multiple myeloma, and renal carcinoma have shown that the expression of PCDGF/GP88 is low in non-tumorigenic cells or in benign lesions and increases in cancer cells in a positive correlation with increased tumorigenic properties and with parameters of poor prognosis. These data and the fact that inhibition of PCDGF expression by antisense transfection and neutralization of PCDGF activity by anti-PCDGF treatment led to inhibition of cell proliferation and in vivo tumorigenesis makes PCDGF as an important target for the development of novel cancer therapy and diagnosis.

Acknowledgments

The work about PCDGF in breast cancer cells and multiple myelomas was supported by Grants RO1 CA 85367 from the National Institutes of Health, DAMD 17-01-1-0550 and DAMD 17-01-1-0551 from the Department of Defense, and Grants 9857-AFF and BCTR2000-356 from the Susan G. Komen Breast Cancer Foundation. The author expresses her gratitude to the Harry and Dorothy Davis family for their generous support of this work in memory of Dorothy Baines Davis. The author thanks the students, post-doctoral fellows, and colleagues who have participated in the cancer studies described here and Dr. Jun Hayashi for reading the manuscript.

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Expression of PC-Cell Derived Growth Factor in Benign and Malignant Human Breast Epithelium

AQ: 1

AQ: 2 GINETTE SERRERO, MD, AND OLGA B. IOFFE

PC cell–derived growth factor (PCDGF, progranulin) is a novel autocrine growth factor that is overexpressed in human breast cancer cell lines. We have examined immunohistochemical PCDGF expression in 206 paraffin-embedded human breast lesions and investigated its association with clinicopathological variables. PCDGF staining was observed in breast carcinoma, whereas it was almost always negative in benign breast epithelium. PCDGF expression was more common in invasive ductal carcinoma (80% cases positive) than in invasive lobular carcinoma (53% positive). PCDGF staining was almost never observed in lobular carcinoma in situ. Ductal carcinoma in situ expressed PCDGF in 66% of the cases, and this expression correlated strongly with nuclear grade. Similar correlation was observed between PCDGF expression and histologic grade of invasive ductal carcinoma. Average Ki-67 index of PCDGF-negative/weakly positive invasive carcinomas (30.3) was significantly lower than that of strongly PCDGF-positive tumors (48.8, $P = 0.01$). A larger percentage of tumors that expressed PCDGF with a staining intensity of 2+ or 3+ were p53 positive (44%) than were PCDGF-negative tumors (25%), $P = 0.02$. PCDGF expression was independent of c-erbB-2 overexpression and of ER and PR status. Our study provides the first evidence of high incidence of PCDGF expression in human breast cancer in which it correlates with clinicopathological variables such as tumor grade, proliferation index, and p53 expression. These characteristics, as well as the virtual absence of expression in benign breast tissue, suggest an important role of PCDGF in breast cancer pathogenesis and make it a potential novel target for the treatment of breast cancer. HUM PATHOL 34:000-000.

Key words: **PC-derived growth factor, breast cancer, immunohistochemistry, prognostic markers, Ki-67, p53.**

Abbreviations: **PCDGF, PC-cell–derived growth factor; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; E_2, 17-β estradiol.**

PC cell–derived growth factor (PCDGF) is an 88-kDa glycoprotein originally purified from the highly tumorigenic mouse teratoma-derived cell line PC.[1,2] PCDGF (also known as progranulin) is the largest member of a novel family of cysteine-rich polypeptides that include the 6-kDa epithelins or granulins that have been shown to either promote or inhibit cell growth depending on the cell type tested.[3,4] Our laboratory was the first to demonstrate the biological activity of PCDGF as a growth promoter for the tumorigenic teratoma PC cells.[2] Others later demonstrated growth-promoting activity of the precursor for other mesenchymal and epithelial cells as well as for preimplantation embryos.[5-7]

Screening of human tumor cell lines for PCDGF expression indicated that it was highly expressed in estrogen receptor–negative (ER−) human breast carcinomas, whereas it was barely detectable in the nontumorigenic immortalized mammary epithelial cells.[8] Inhibition of PCDGF expression by antisense PCDGF cDNA transfection in ER− human breast carcinoma resulted in a dramatic reduction (>98%) in tumor incidence and tumor size when injected in nude mice,[8] implicating PCDGF as a major factor in the maintenance of tumor phenotype. In ER-positive (ER+) cells, PCDGF expression was stimulated by 17-β estradiol (E_2) and inhibited by tamoxifen.[9] Recently, we demonstrated that PCDGF mediated E_2 mitogenic effect in ER+ breast cancer cells,[10] whereas PCDGF overexpression rendered the cells able to proliferate in the absence of estrogen, although estrogen receptor expression of the cells remained unchanged.[10] These various studies pointed out the importance of PCDGF for the proliferation of breast cancer cells and suggested that increase of PCDGF expression played a major role in the maintenance of the breast cancer phenotype. On the basis of these observations, the present study was carried out to investigate PCDGF expression in human breast cancer. PCDGF expression was determined by immunohistochemical staining using an anti-human PCDGF antibody in formalin-fixed, paraffin-embedded human breast tissue. PCDGF expression in invasive tumors was examined in association with clinicopathological variables such as the histologic grade and type; expression of estrogen and progesterone receptors (ER/PR); as well as other markers such as proliferation rate (Ki67 index), p53, and c-erbB-2.

Ki-67 (MIB-1) is a protein vital to cell proliferation; it is a nuclear antigen that is expressed in all phases of the cell cycle except in G_1 or G_0 phase,[11] and it is recognized by the antibody MIB-1 in paraffin-embedded archival tissue. High Ki-67 index has been shown to

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TABLE 1. Cases Included in the Analysis

Diagnosis	# of cases (%)
IDC	124
Grade 1	36 (29)
Grade 2	40 (32.3)
Grade 3	48 (38.7)
DCIS	27
Low nuclear grade	8 (29.7)
Intermediate nuclear grade	3 (11)
High nuclear grade	16 (59.3)
ILC	17
LCIS	12
Benign	26

correlate with shortened disease-free survival in breast cancer on multivariate analysis[12] and with shortened overall and disease-free survival.[13]

c-erbB-2 (*HER2/neu*) is an oncogene that encodes a 185-kDa ligandless receptor tyrosine kinase belonging to the epidermal growth factor receptor superfamily. Its overexpression (seen in 20% to 30% of breast cancers) has been linked to poor outcome, especially in node-positive patients, whereas its effect on prognosis in node-negative disease has remained controversial (reviewed in Ross and Fletcher[14]).

p53 is a tumor suppressor gene that is involved in cell cycle arrest. In breast cancer, it detects high-risk patients, especially if they are node-negative. *p53* mutations, which can be identified immunohistochemically, are linked to poor prognosis, high histologic grade and proliferation rate, aneuploidy, and steroid receptor negativity.[15]

MATERIALS AND METHODS

Tissue Samples

Two hundred six nonconsecutive archival formalin-fixed, paraffin-embedded human breast lesions from 152 patients were obtained from the University of Maryland Department of Pathology files and from the National Cancer Institute of Canada–Manitoba Breast Cancer Tumor Bank of the University of Manitoba, Winnipeg, Canada (kindly provided by Dr. Peter Watson). The 152 tissue blocks examined contained the following lesions: 27 ductal carcinoma in situ (DCIS), 12 lobular carcinoma in situ (LCIS), 124 invasive ductal carcinoma (IDC), 17 invasive lobular carcinoma (ILC), all classic variant, and 26 benign unremarkable breast tissue. Special-type mammary carcinomas other than invasive lobular carcinoma were not included in this study. The DCIS cases were graded according to nuclear grade as defined by the European Breast Screening Group.[16,17] The Elston (Nottingham) grading system[18] was used for the determination of histologic grade of IDC. Histologic grading was performed by the pathologist of the study (OBI). The composition, histologic grade, and type of the cases examined in this study are shown in Table 1.

Steroid hormone receptor status data were available for most invasive carcinoma cases and had been determined by biochemical method (Dextran-coated charcoal);[19,20] the biochemical tests for ER and PR were considered negative if the results were <3.0 and <10.0 fmol/mg cytosol protein, respectively.

Immunohistochemistry

Four-micrometer sections were cut from a representative paraffin block in each case; these sections were immunostained using a standard peroxidase-conjugated streptavidin biotin method. The tissue sections were dewaxed and rehydrated. Antigen retrieval was performed using DAKO target retrieval solution (DAKO Corporation, Carpinteria, CA). Immunostaining was performed using Ventana autostainer (Ventana, Tucson, AZ). The slides were counterstained with hematoxylin. Appropriate positive and negative controls were included in each run.

Detection of PCDGF by Immunohistochemistry

PCDGF was detected in tissue sections by immunostaining using an immunoaffinity purified anti-human PCDGF rabbit polyclonal antibody (1 μg/mL). Purity and specificity of the antibody had been previously determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. On Western blot analysis of cell lysates, this antibody recognized a single 88-kDa band that could be competed by preincubation of the antibody with excess antigen as described elsewhere.[10] PCDGF expression was cytoplasmic and granular and was semiquantitatively categorized as follows: <5% of cells staining, negative; >5% of cells staining, positive; positive staining was graded from weak/focal (1+) to moderate/focal or diffuse (2+) to strong/diffuse (3+).

Detection of Ki-67, P53, and C-erbB2

The proliferation rate was measured by determining the Ki-67 index. Detection of Ki67 was carried out by immunostaining using anti-MIB-1 mouse monoclonal antibody (DAKO). Ki67 index was expressed as the percentage of positively staining nuclei per 1000 cells counted. Immunostaining for p53 was performed by using an anti-p53 mouse monoclonal antibody from BioGenex Laboratories (San Ramon, CA). Expression of p53 was categorized as follows: <10% of nuclei staining, negative; >10% of nuclei staining, positive.[21] C-erbB-2 immunostaining was performed using anti-c-erbB-2 rabbit antibody (DAKO). C-erbB-2 expression was assessed by the presence and intensity of the cell membrane staining as follows: <10% staining, negative; >10% cells staining, positive; positive staining was graded from focal, weak, discontinuous membrane staining (1+); to focal, moderate, continuous membrane reactivity (2+); to homogeneous, strong, and continuous membrane positivity (3+).

Statistical Analysis of the Data

Statistical analysis of the data was performed using the analysis of variance method, F test for variances, regression test, and χ^2 test. P values of <0.05 were considered statistically significant. The Minitab, Inc statistical software package, version 13.1, was used for statistical analysis.

RESULTS

PCDGF Expression in Human Breast Tissue and Its Association With Histologic Type

PCDGF expression was observed in 128 of 206 cases (62%; Table 2). Most benign breast epithelium was negative for PCDGF (25 of 26 cases, or 96%), as were most LCIS cases (11 of 12, or 92%). However, the

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TABLE 2. Results of PCDGF Immunostaining

Diagnosis	# of cases	PCDGF staining, n (%) Negative	Weak (1+)	Moderate/strong (2+/3+)
Benign	26	25 (96)	1 (4)	0
DCIS	27	9 (33)	8 (30)	10 (37)
LCIS	12	11 (92)	1 (8)	0
IDC	124	25 (20.2)	48 (38.8)	51 (41)
ILC	17	8 (47)	6 (35)	3 (18)

NOTE: The difference in PCDGF expression between benign/LCIS and intraductal/invasive carcinomas ($P = 0.001$) and between invasive ductal and lobular carcinomas ($P = 0.03$) is statistically significant.

majority of malignant noninvasive and invasive ductal lesions showed PCDGF expression. The difference in PCDGF expression between benign/LCIS and intraductal/invasive carcinomas was statistically significant ($P = 0.001$). Eighteen of the 27 DCIS (67%) and even a higher proportion of IDC, 99 of 124 (79.8%), expressed PCDGF. ILC expressed PCDGF in about half of the cases: 9 of 17 (53%). There was a striking difference in the degree of PCDGF expression between invasive ductal and lobular carcinomas. Whereas 51 of 124 (41%) cases of IDC showed moderate or strong PCDGF reactivity (2+ or 3+), only 3 of 17 (18%) ILC were moderately or strongly positive ($P = 0.03$). Figure 1A shows strong (3+) PCDGF staining in IDC. The staining was confined to the cytoplasm; it was coarsely to finely granular. The typical absence of PCDGF expression in in situ and invasive lobular carcinomas is shown in Fig 1B and C.

F1

PCDGF Expression and Histologic Grade of Ductal Carcinoma

As shown in Tables 3 and 4, PCDGF expression showed a strong correlation with the histologic grade of both in situ and invasive ductal carcinomas. The majority of low–nuclear grade DCIS were negative for PCDGF

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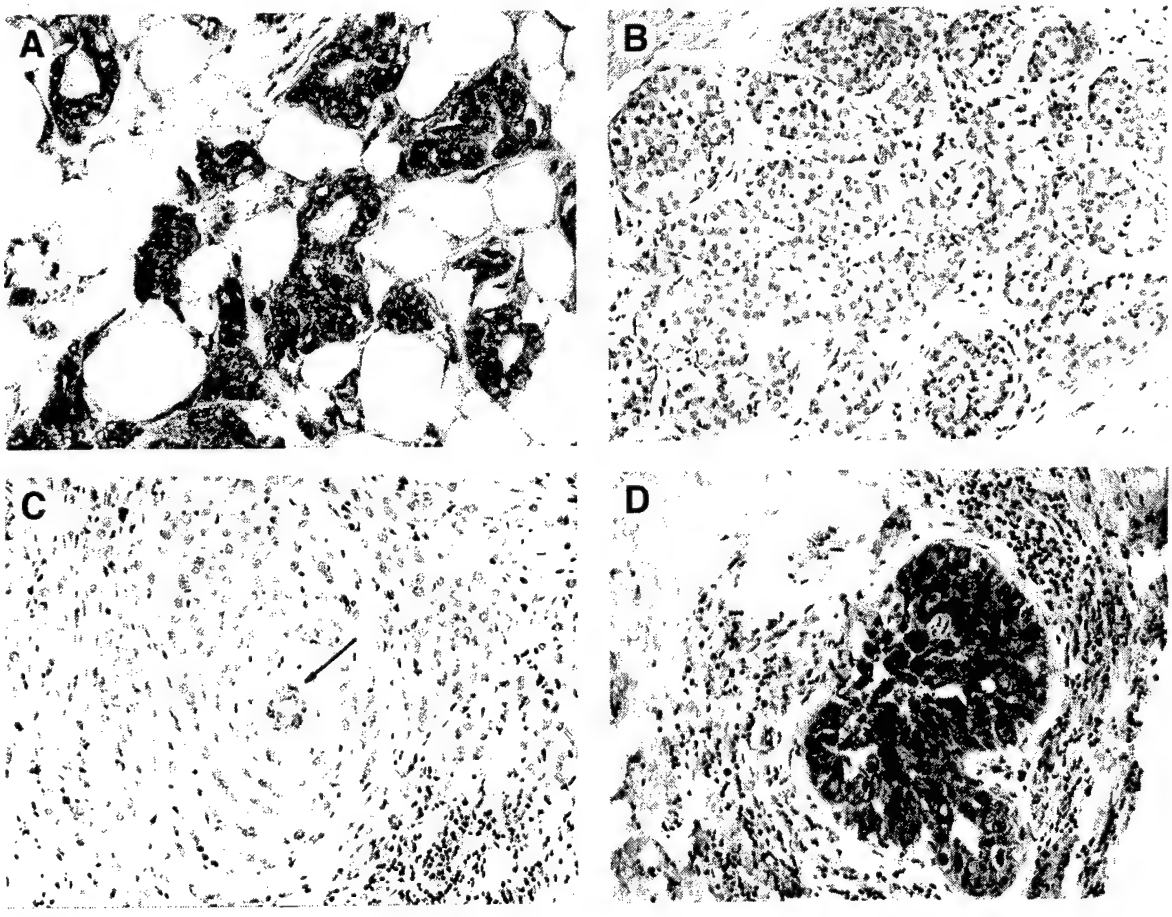

FIGURE 1. Immunostaining for PCDGF in paraffin- embedded human breast tissue. (A) Strong (3+) diffuse cytoplasmic reactivity for PCDGF is seen in this invasive ductal carcinoma. (B) Lobular carcinoma in situ shows no PCDGF expression. (C) Invasive lobular carcinoma lacking PCDGF reactivity. Benign duct (arrow) is also negative for PCDGF. (D) Ductal carcinoma in situ, high nuclear grade, solid type strongly expresses PCDGF (3+).

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TABLE 3. PCDGF Staining In Ductal Carcinoma In Situ (DCIS)

Nuclear grade of DCIS	PCDGF staining, n (%) Negative (0)	Weak (1+)	Moderate (2+)	Strong (3+)
Low grade (n = 16)	9 (56)	7 (44)	0	0
Intermediate grade (n = 3)	0	0	3 (100)	0
High grade (n = 8)	0	1 (12.5)	3 (37.5)	4 (50)

NOTE: Immunohistochemical expression of PCDGF significantly correlates with nuclear grade of DCIS ($P < 0.0001$). This significant difference is seen between low and high nuclear grade cases.

(9 of 16, or 56%), and all the remaining cases were weakly (1+) positive. All DCIS of intermediate nuclear grade (3 of 3, or 100%) showed moderate PCDGF positivity (2+), and most high-grade DCIS (7 of 8, or 87.5%) had moderate or high PCDGF expression (Fig 1D). The difference in PCDGF expression between low- and high-grade DCIS was statistically significant ($P < 0.0001$).

A similar pattern of PCDGF distribution in relation to histologic grade was seen in IDC. All grade 1 tumors were either negative or weakly positive for PCDGF. In contrast, 47.5% (19 of 40) of grade 2 tumors showed moderate to strong PCDGF reactivity. Only 5 (12%) grade 2 IDC were negative for PCDGF; moreover, none of the 48 grade 3 carcinomas lacked PCDGF expression, and two thirds (32 of 48, or 67%) showed moderate to strong immunoreactivity for PCDGF. The differences in PCDGF staining in all 3 histologic grades were statistically significant: between grade 1 and 2 ($P = 0.01$), grade 1 and 3 ($P < 0.0001$), and grade 2 and 3 ($P = 0.07$).

PCDGF Expression and Proliferation Rate of Invasive Carcinomas

The proliferative rate as expressed by Ki-67 index showed a significant correlation with PCDGF expression in all invasive carcinomas (Fig 2). The average Ki-67 index of PCDGF-negative carcinomas was 30.3. The Ki-67 index was 32.7 in weakly positive tumors, 34.8 in cancers that were moderately (2+) PCDGF positive, and 48.8 in strongly (3+) PCDGF-positive tumors. The Ki-67 index of the tumors that were either negative or weakly positive was significantly lower than that of the moderately and strongly PCDGF-positive carcinomas ($P = 0.01$).

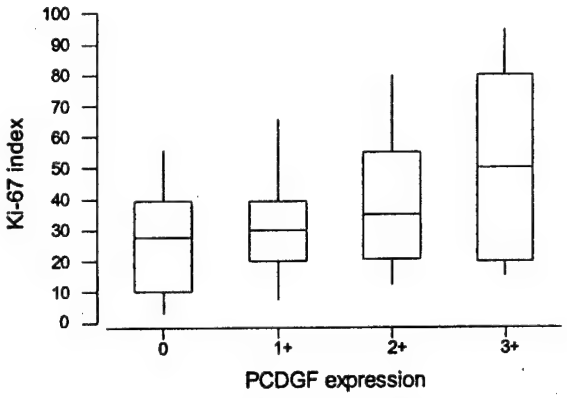

FIGURE 2. Box plot of Ki-67 index (% of positive cells) and PCDGF expression (graded from 0 to 3+) In invasive breast carcinomas. The difference between negative/weak and strong PCDGF expression is statistically significant ($P = 0.01$).

PCDGF Expression and ER and PR Status

Of 109 invasive carcinomas for which the ER and PR status was available, 37 (34%) were positive for both ER and PR. Thirty-six tumors (33%) were positive for ER and negative for PR, and 36 (33%) were negative for both receptors. No cases negative for ER and positive for PR (ER−/PR+) were seen in this study. It has been previously reported that ER−/PR+ tumors are relatively rare, accounting for only 4% of all breast cancers.[22] The ER and PR status was compared with the PCDGF expression of the same invasive tumors (Table 5). Of the 65 invasive cancers that were negative or weakly positive for PCDGF expression, 24 (37%) were positive for ER and PR, and 22 (30.7%) were negative for ER and PR. Thirteen of 44 (29.6%) moderately to strongly PCDGF-expressing cancers were ER and PR positive, whereas 14 of 44 (31.8%) were negative for ER and PR ($P = 0.1$).

TABLE 4. PCDGF Staining In Invasive Ductal Carcinoma (IDC) According to Its Histologic Grade

Histologic grade of IDC	PCDGF staining, n (%) Negative (0)	Weak (1+)	Moderate (2+)	Strong (3+)
Grade 1 (n = 36)	20 (55.5)	16 (44.5)	0	0
Grade 2 (n = 40)	5 (12.5)	16 (40)	13 (32.5)	6 (15)
Grade 3 (n = 48)	0	16 (30)	24 (50)	8 (20)

NOTE: PCDGF expression shows a significant correlation with histologic grade of IDC: the difference is significant between grade 1 and 2 ($P = 0.01$), grade 1 and 3 ($P < 0.0001$), and grade 2 and 3 ($P = 0.07$).

TABLE 5. PCDGF Expression and Steroid Receptor Status In All Invasive Carcinomas

ER/PR status	PCDGF staining, n (%) Negative (0)	Weak (1+)	Moderate/strong (2+/3+)
ER+/PR+ (n = 37)	6 (16)	18 (49)	13 (35)
ER+/PR− (n = 36)	4 (11)	15 (42)	17 (47)
ER−/PR+ (n = 0)	0	0	0
ER−/PR− (n = 36)	10 (28)	12 (33)	14 (39)

No significant difference is observed between ER/PR status and PCDGF expression of invasive carcinomas ($P = 0.1$).

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TABLE 6. Comparison of PCDGF Staining and p53 Expression in Invasive Breast Carcinoma

P53 expression	PCDGF staining, n (%)		
	Negative (0)	Weak (1+)	Moderate/strong (2+/3+)
Negative (n = 83)	26 (32)	29 (35)	28 (33)
Positive (n = 40)	8 (20)	10 (25)	22 (55)

NOTE: Immunohistochemical expression of PCDGF significantly correlates with p53 reactivity between all categories ($P = 0.08$), as well as between cases with 0/1+ versus 2+/3+ PCDGF expression ($P = 0.02$).

PCDGF Expression and P53 Status

Among the 73 cases of invasive carcinomas that were negative or weakly positive for PCDGF, 18 (25%) tumors were p53 positive. Of the 50 PCDGF 2+/3+ cases, 22 (44%) exhibited p53 reactivity; 18 of 73 (25%) PCDGF 0/1+ cases were positive for p53 ($P = 0.02$; Table 6). Therefore, p53 expression was significantly more common in carcinomas that strongly expressed PCDGF.

PCDGF Expression and C-erbB-2 Status

Among the cases of invasive carcinoma that were negative or weakly positive for PCDGF, 46 of 80 (58%) were negative or weakly positive (1+) for c-erbB-2, 15 (18%) were moderately (2+) positive for c-erbB-2, and 19 (24%) were strongly c-erbB-2–positive (3+; Table 7). Of the cases moderately to strongly expressing PCDGF (2+/3+), 31 of 53 (58.5%) did not show immunohistochemical evidence of c-erbB-2 overexpression (0/1+ c-erbB-2 staining), and 13 of 53 cases (24.5%) had strong c-erbB-2 reactivity (3+; $P = 0.9$). These results indicate that there is no correlation between c-erbB-2 and PCDGF expression in the invasive mammary carcinomas examined.

DISCUSSION

Our studies with human breast cancer cell lines have indicated the biological importance of PCDGF in breast cancer tumorigenesis,[8-10] thereby warranting the investigation of its expression in archived pathological samples. This is the first report describing the expression of PCDGF in human breast tissue. We have shown that PCDGF is almost never expressed in benign breast epithelium. PCDGF expression was not seen in lobular carcinoma in situ, whereas the majority of invasive lobular carcinoma cases (82%) were either negative or weakly positive. PCDGF expression was found in most cases of in situ and invasive ductal carcinoma. PCDGF was expressed in 80% of IDC, with a staining of 2+ or greater in 41% of the cases. Therefore, there appears to be a preferential expression of PCDGF in ductal rather than lobular lesions. In malignant ductal lesions, the degree of PCDGF staining correlates with the histologic grade in both DCIS and IDC. These data support the fact that in human breast cancer cell lines, the level of PCDGF expression in breast carcinoma cell lines appears to be positively correlated to their tumorigenesis.[8]

Interestingly, PCDGF staining intensity in invasive carcinomas correlated with the proliferation (Ki-67) index in our study. Growth fraction determined by Ki-67 in breast carcinomas closely correlates with many other indicators of proliferation, such as S-phase by flow cytometry,[23] thymidine labeling index,[24] BrdU index, and mitotic counts.[25] The fact that PCDGF expression correlated with Ki67 index is important because Ki-67 index is an independent factor of poor prognosis, especially in node-negative patients.[13,25] We have previously shown that PCDGF is a growth stimulator of several breast cancer cell lines and that inhibition of PCDGF expression by antisense transfection or action by neutralizing antibodies resulted in inhibition of in vitro cell proliferation and in vivo tumorigenesis.[8,10] The finding that PCDGF staining in the current study was significantly associated with Ki-67 index is in agreement with the fact that PCDGF is a growth stimulatory factor for breast cancer.

The correlation of PCDGF expression with histologic grade most likely stems from the growth-stimulating properties of PCDGF since it has been shown that the proliferative rates correlate with histologic grade of invasive and in situ ductal carcinomas.[26-28]

Our study would suggest that ER and PR status does not correlate with PCDGF expression. It has been shown that ER and PR-negative tumors tend to have higher proliferation rates.[29] It is possible that PCDGF contributes to the stimulation of proliferation independently of steroid receptors. In fact, our previous studies have shown that even though a higher level of PCDGF expression was found in ER-negative breast cancer cells,[8] overexpression of PCDGF in ER-positive MCF-7 cells led to estrogen independence without change of ER expression status.[10] It is also important to note that the steroid receptor status of cases used in this study had been determined by the biochemical method. Although biochemical and immunohistochemical methods have demonstrated concordance in 77% to 81% of cases,[30,31] immunohistochemical method is considered to be more accurate in predicting response to hormone therapy;[32,33] some of the reasons for this are the sampling error (tissue not containing tumor, scant tumor

TABLE 7. Comparison of PCDGF Staining and C-erbB-2 Expression in Invasive Breast Carcinoma

C-erbB-2 staining	PCDGF staining, n (%)		
	Negative (0)	Weak (1+)	Moderate/strong (2+/3+)
0/1+ (n = 77)	18 (23)	28 (37)	31 (40)
2+ (n = 24)	5 (21)	10 (42)	9 (37)
3+ (n = 32)	6 (18)	13 (41)	13 (41)

NOTE: There is no significant difference between c-erbB-2 expression and PCDGF reactivity ($P = 0.9$).

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cellularity or significant admixture of benign breast epithelium) and occupation of steroid receptor by endogenous estradiol. We plan to obtain immunohistochemical ER and PR status for all cases in our series and reassess the correlation between PCDGF expression and steroid receptor status.

Our study showed that PCDGF expression correlates with p53 immunoreactivity, which is an accepted indicator of the presence of *p53* mutation. P53 positivity has been shown to be associated with poor outcome, especially in lymph node–negative breast cancer,[15] and it is an independent prognostic marker. Interestingly, p53 has been shown to directly correlate with Ki-67 index and not with c-erbB-2 status,[34] similar to PCDGF in our study.

Because PCDGF and c-erbB-2 are both implicated in the activation of growth-promoting signaling pathways, the lack of significant association between PCDGF and c-erbB-2 in the cases studied here is interesting. C-erbB-2 overexpression, almost always the result of gene amplification, is associated with increased tumor growth rate, enhanced metastatic rate, and shorter disease-free and overall survival rate,[35,36] although c-erbB-2 status has not been consistently proven to represent an independent prognostic indicator.[37] The lack of correlation between PCDGF and c-erbB-2 expression is in agreement with results of our previous studies of human breast cancer cell lines, in which we have shown that high PCDGF expression could be observed in cells that did not express c-erbB-2 (MDA-MB-468 cells) as well as in cells reported to express c-erbB-2 such as MDA-MB-453.[8] The fact that PCDGF is overexpressed in IDC that are negative or weakly positive for c-erbB-2 would suggest that these two growth factor signaling pathways might be distinct and activated independently from each other. This possibility is presently investigated in detail in our laboratory.

Co-expression of epidermal growth factor (EGF) receptors and transforming growth factor alpha (TGF-α) has been shown to constitute an adverse prognostic factor for breast cancer patients.[38,39] Although no correlation was found between PCDGF and c-erbB-2 expression, it should be pointed out that 24.5% of the cases examined were strongly positive for both PCDGF and c-erbB-2. Experiments are currently underway to investigate the prognostic significance of PCDGF expression, either alone or in combination with c-erbB-2 in breast cancer.

The difference in PCDGF expression in invasive lobular and ductal carcinomas is of considerable interest. Although the prognosis of IDC and ILC is essentially identical if matched by stage,[40] the proliferation rates of ILC as measured by mitotic index, Ki-67 index, AgNOR measurement[25,41] as well as S-phase fraction determination by flow cytometry[42] have been reported to be significantly lower than those of IDC. Because we have shown elsewhere that PCDGF is a growth stimulator,[8,10] the relative lack of PCDGF expression in ILC certainly correlates with the low proliferation rates of these tumors. LCIS have been reported to have extremely low proliferation rates,[41,43] similar to those of benign breast epithelium. These findings would certainly correlate with the lack of PCDGF expression in both benign breast epithelium and LCIS.

In summary, our studies provide the first direct evidence of high incidence of PCDGF expression in human breast cancer, in which it correlates with such clinicopathological variables as tumor grade, proliferation index and p53 expression. These characteristics, along with the lack of expression in benign epithelium, and considering our previous studies in breast cancer cell lines suggest the important role of PCDGF in breast cancer and make it a potential target for the development of novel therapy for the treatment of breast cancer. Correlation with survival and other clinical variables is currently under investigation.

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PC-Cell Derived Growth Factor (PCDGF/progranulin) mediates tamoxifen resistance and promotes tumor growth of human breast cancer cells.

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Abstract

PC Cell Derived Growth Factor, also known as progranulin is an 88 kDa growth factor (referred as PCDGF/GP88) overexpressed in human breast cancer. Antisense inhibition of PCDGF/GP88 expression in MDA-MB-468 cells inhibited tumor formation in nude mice. In estrogen receptor positive cells, PCDGF/GP88 was expressed in response to estradiol and shown to mediate its mitogenic effect. Pathological studies indicated that PCDGF/GP88 was expressed in 80% of invasive ductal carcinoma in correlation with parameters of poor prognosis. In the present paper, the relationship between PCDGF/GP88 expression and tamoxifen resistance was examined in MCF-7 cells. PCDGF/GP88 overexpression rendered MCF-7 cells able to proliferate in the absence of estrogen and in the presence of tamoxifen. The PCDGF/GP88 overexpressing cells formed tumors in ovariectomized nude mice in the absence of estradiol as well as in its presence, in contrast to MCF-7 cells. Tumor growth of the overexpressing cells was significantly increased when the mice were treated with tamoxifen. PCDGF/GP88 blocked tamoxifen-induced apoptosis by preventing down-regulation of bcl-2 expression and PARP cleavage. In addition, PCDGF/GP88 overexpressing cells presented higher level of the angiogenic factors VEGFs and angiopoietin-1 than MCF-7 control cells. Tamoxifen treatment further increased the level of VEGF. These studies suggest that PCDGF/GP88 plays a critical role in breast cancer tumorigenesis and in the transition to estrogen independence and tamoxifen resistance, a hallmark of poor prognosis. Based on the in vivo studies, it is postulated that tamoxifen treatment of patients with ER^+ breast tumors overexpressing PCDGF/GP88 could have adverse clinical consequences.

Introduction

Anti-estrogen therapy is widely used for the treatment of breast cancer because it is well tolerated unlike conventional cytotoxic chemotherapy regimens. Tamoxifen has been the major agent used for this purpose until the recent development and clinical application of novel estrogen receptor antagonists such as ICI 182,780 (1). The inhibitory effect of tamoxifen is observed almost exclusively in breast tumors that are estrogen receptor positive (ER^+), since estrogen is the major growth stimulator for these types of tumors. However, after prolonged anti-estrogen hormonal therapy, breast cancer often progresses from an estrogen sensitive to insensitive state (2). In this case, the growth of breast tumors that was previously inhibited by tamoxifen becomes refractory to tamoxifen treatment. Although the development of tamoxifen resistance may be associated with the acquisition of an estrogen receptor negative phenotype, tamoxifen resistance has also been observed in estrogen receptor positive tumors (3, 4). In these later cases, constitutive overexpression of autocrine growth factor, or growth factor receptor by tumor cells, has been proposed as one possible mechanism for developing tamoxifen resistance (5). Such increased autocrine or paracrine growth factor signaling network could then bypass the need for ER-mediated growth stimulation in human breast cancer cells and would render anti-estrogen therapy ineffective. For example, clinical studies have reported a decreased efficacy of tamoxifen for tumors overexpressing c-erbB2 (6). In addition to inhibiting the growth promoting effect of estrogen, tamoxifen has also been shown to induce programmed-cell death in breast cancer cell lines and in clinical samples (7-9). Failure to undergo apoptosis in response to tamoxifen would also confer tamoxifen resistance (10). Therefore, increase in autocrine growth factor signaling that mediates both proliferation signals and anti-apoptotic signals may induce resistance to tamoxifen therapy.

PC-Cell Derived Growth Factor (PCDGF/GP88) is an 88 kDa cysteine rich glycoprotein originally purified as an autocrine growth factor from the conditioned medium of the highly tumorigenic mouse teratoma PC cells (11). PCDGF/GP88, also known as granulin/epithelin precursor or progranulin, has been found to be overexpressed in many cancer cells including teratoma (12), breast cancer (13), ovarian cancer (14), renal carcinoma (15), multiple myeloma (16) and glioblastoma (17). It has been reported that PCDGF/GP88 stimulates proliferation and survival in several cell types including cancer cells as well as fibroblasts, endothelial cells and pre-implantation embryos (18). These biological activities are mediated via activation of mitogen activated protein kinase and phosphatidylinositol 3-kinasepathways (for review (19). In human breast cancer cells, PCDGF/GP88 expression was stimulated by estradiol in a time- and dose-dependent fashion in estrogen receptor positive cells (13). In these cells, PCDGF/GP88 was shown to mediate the mitogenic activity of estrogen by stimulating cyclin D1 expression (20). Inhibition of PCDGF/GP88 expression in estrogen receptor negative MDA-MB-468 cells by antisense transfection led to a complete inhibition of tumorigenesis in nude mice (21). Pathological studies in paraffin embedded breast cancer biopsies have shown that high PCDGF/GP88 expression was found in 60 % of ductal carcinoma *in situ* (DCIS) and 80% of invasive ductal carcinoma (IDC) whereas normal mammary epithelium and benign tumors tested negative for PCDGF/GP88 (22). In IDC, PCDGF/GP88 expression correlated with parameters of poor prognosis such as tumor grade, proliferation index and p53 positivity. Correlation studies with estrogen receptor expression in the biopsies indicated that PCDGF/GP88 expression was found in ER positive as well as ER negative tumors (22). Interestingly, 20% of ER positive IDCs expressed high level of PCDGF (21). In the present paper, we examined the effect of PCDGF/GP88 overexpression in estrogen receptor positive breast cancer cells and investigated the possible correlation

between PCDGF/GP88 overexpression and tamoxifen resistance in human breast cancer cells. We used PCDGF/GP88 overexpressing MCF-7 cells as models to determine the effect of PCDGF/GP88 on responsiveness of the cells to tamoxifen *in vitro* and in mouse xenograft models. Our results indicate that the overexpression of PCDGF/GP88 confers tamoxifen resistance, prevents tamoxifen-induced apoptosis in MCF-7 cells and, lastly, promotes tumor growth and angiogenesis in the presence of tamoxifen *in vivo*.

Material and Methods

Materials:

17β-Estradiol (E2) was purchased from Calbiochem (San Diego, CA). G418, Taq polymerase and Superscript II were obtained from Gibco BRL (Gaithersburg, MD). Tamoxifen was purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotide primers used in the RT-PCR were synthesized by the Biopolymer Core Laboratory of the University of Maryland (Baltimore, MD). E2 and placebo pellets were obtained from Innovative Research of America (Sarasota, Fl). Enhanced chemiluminescence kit was obtained from Pierce (Rockford, IL). Mouse anti-poly (ADP-ribose) polymerase antibody (anti-PARP) was purchased from Oncogene Research (Boston, MA).

Cell proliferation assay

The ER^+ human breast cancer cell line MCF-7 was obtained from ATCC (Manassas, VA). PCDGF/GP88 overexpressing MCF-7 cells (O4 and O7 cells) were developed as previously described (20). Control MCF-7 cells (MCF-7 EV) were obtained by transfecting empty pcDNA3 vector and selecting G418 resistant cells as described previously(20). Both cell lines were maintained in DMEM/F12 supplemented with 5% FBS, and 400 μg/ml of G418. For proliferation assay, 5×10^4 cells were plated in 6-well culture plates (Costar, Cambridge, MA) in phenol red-free α-MEM (PFMEM) supplemented with 5% charcoal extracted fetal bovine serum (CHX-FBS). Cells were treated with either 1 nM E2 alone or in combination with increasing doses of tamoxifen. Control cells were treated with vehicle alone (0.01% DMSO). Medium was changed at day 4. Cell numbers were counted by hemocytometer. Each time point was performed in triplicate.

[^{3}H]-Thymidine incorporation assay.

Cells were inoculated at a density of 4 x 10^4 cells per well on 24-well dishes in DMEM/F12-supplemented with 5% FBS. After 24 hours of incubation, the medium was changed to PFMEM, supplemented with 5% CHX-FBS. After 24 hours, cells were washed once with medium and medium was changed to serum-free PFMEM. The cells were treated for 24 hours either with vehicle only, 1 nM of E2 alone or in combination with 1 μM tamoxifen. Thymidine incorporation assay was performed in triplicate as described previously (13).

***In vivo* tumorigenesis assay in nude mice**

MCF-7 EV or O4 cells (5 x 10^6 cells per site) were injected subcutaneously in two sites into six-week-old ovariectomized athymic female nude mouse (National Cancer Institute, Frederick, MD). E2 pellets (1.7mg-60 day release) or placebo pellets were implanted subcutaneously in the back, one day before inoculating the cells. Five to ten nude mice per experimental group were used depending on the experiments. For the *in vivo* tamoxifen resistance experiment, the animals that had received E2 pellets and been injected with the cells were implanted with tamoxifen base pellets (5 mg, 60-day release) or placebo pellets, ten days after the cell inoculation. The width (W) and length (L) of individual tumors were measured weekly with a caliper. Average tumor volume was calculated with the widely used formula: tumor volume =(width^2 x length) x 0.5. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Measurement of ERE-luciferase reporter gene activity

MCF-7 EV or O4 cells (2.5 x 10^5 cells) were plated in PFMEM supplemented with 5% FBS in 6-well plates. Cells were transiently transfected with pGL2-ERE-luciferase plasmid DNA

by lipofectAMINE (Gibco, Gaithersburg, MD). pcDNA3-β-galactosidase construct was co-transfected as an internal control to determine transfection efficiency. One nM E2 and/or 1 μM tamoxifen was added 5 hours after transfection. Cell lysates from triplicate dishes were collected using reporter lysis buffer after 36 h of transfection. Determination of luciferase and β-galactosidase activities was performed using kits following manufacturer's protocols (Promega, Madison, WI). Luciferase activity values were normalized to the transfection efficiency in each condition, determined by measuring β-galactosidase activity of each sample.

Determination of mRNA expression for bcl-2, bcl-x_L, bax, VEGF, angiopoietin-1 and 2 by RT-PCR

Five micrograms of total RNA were reverse transcribed into single strand cDNA by Super Script II (Gibco) using 250 ng random hexamer (Gibco) as primer. The RT reaction was carried out for 1 h at 42 C in 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, DTT 0.01 M and dNTP (each 0.5 mM). A total of 30-35 PCR cycles depending on the gene amplified was performed, followed by electrophoresis on 1% agarose gel. The specific primer pairs used were:

for glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer 5' TGAAGGTCGGAGTCAACGGATTTGGT 3', reverse primer, 5' CATGTGGGCCATGAGGTCCACCAC 3';

for bcl-2: forward primer 5' GGTGCCACCTGTGGTCCACCTG 3', reverse primer 5' CTTCACTTGTGGCCCAGATAGG 3';

for bax: forward primer 5' GAGCAGATCATGAAGACAGGGG 3', reverse primer 5' CTCCAGCAAGGCCCAGCGTC 3';

for bcl-x_L: forward primer 5' CAGTGAGTGAGCAGGTGTTTTGG 3', reverse primer 5' GTTCCACAAAAGTATCCCAGCCG 3';

VEGF: forward primer 5' ATGAACTTTCTGCTGTCTTGGGT 3', reverse primer 5' TCACCGCCTCGGCTTGTCAC 3'

for angiopoietin-1: forward primer 5' TTGCTTTCCTCGCTGCCATTC 3', reverse primer 5' CAGCATGGTAGCCGTGTGGTTC 3';

for angiopoietin-2: forward primer 5' AGCTACACTTTCCTCCTGCCAG 3', reverse primer 5' AGCCGTCTGGTTCTGTACTGC 3'.

Western Blot analysis of PARP cleavage:

Cells were seeded at a density of 7 x 10^5 cells in 60-mm dish in DMEM/F12 supplemented with 5%FBS. After 24 hours, medium was changed to serum-free phenol red-free DMEM/F12 supplemented with vehicle or purified PCDGF/GP88 (200 ng/ml) for another 24 hours. Cells were treated with either vehicle only or factors under investigation for 24 hours. Cell lysates were collected in RIPA buffer (50 mM Tris- HCl pH 7.4 containing 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1mM NaF, protease inhibitor cocktail) containing 6 M urea. 100 μg of protein from each sample was used for immunoblotting. Intact and cleaved forms of PARP were detected using a mouse monoclonal anti-PARP antibody. The band intensity of PARP cleaved form was determined by densitometric analysis and normalized to the actin level used as internal standard.

Statistical analysis

All experiments were conducted in triplicates and repeated at least twice. Data were analyzed by Student *t* test for mean comparison and statistical significance. The values are reported as mean ± standard error.

Results

Overexpression of PCDGF/GP88 promotes growth and confers tamoxifen resistance *in vitro*

We first compared the effect of increasing doses of tamoxifen on the proliferation of PCDGF/GP88 overexpressing MCF-7 cells (O4 and O7 cells) and the empty vector transfected MCF-7 EV. As shown in Fig. 1*A*, DNA synthesis in PCDGF/GP88 overexpressing cells (O4 and O7) was not affected by tamoxifen, even at doses (1 μM) that inhibited MCF-7 EV proliferation by 90%. The lack of inhibitory effect of tamoxifen on PCDGF/GP88 overexpressing cell growth was confirmed by long term growth assay in estrogen-depleted medium supplemented with or without E2 and tamoxifen (Fig. 1*B*). PCDGF overexpressing cells could proliferate in the absence of E2 (doubling time O4, 42.1 ± 0.7 h; O7, 39.1 ± 1.95; $p>0.05$) as well as in the presence of E2 alone (doubling time O4, 37.3 ± 1.6 h; O7, 35.4 ± 2.2; $p>0.05$) or with 1 μM tamoxifen (doubling time O4, 39.1 ± 1.4 h; O7, 37.4 ± 2.4; $p>0.05$), close to the doubling time of MCF-7 EV cells in the presence of E2 (37.9 ± 1.0 h). Based on these data, all subsequent experiments were carried out with O4 cells as representative of PCDGF/GP88 overexpressing cells

PCDGF/GP88 overexpression mediates estrogen-independent tumor growth *in vivo*

Since PCDGF overexpressing cells were able to proliferate in the absence of E2 *in vitro*, their ability to form tumors in ovariectomized nude mice was examined and compared to MCF-7 EV cells. Tumorigenesis study was carried out in ovariectomized nude mice implanted with either placebo or E2 pellets. Tumor size was measured over a 45-day period and the mean tumor volume was determined as described in the method section. As shown in Fig. 2, O4 cells formed tumors in both placebo and E2 pellets implanted ovariectomized mice whereas

MCF-7 EV cells formed tumors only in mice receiving E2 pellets. The incidence of tumor formation for O4 cells in placebo and in E2 treated ovariectomized mice (70-90%) was higher than the one for MCF-7 EV cells in E2 treated mice (33-40%). The mean tumor volume for O4 cells in mice treated with placebo pellets (62.2 ± 7.0 mm^3) was significantly higher than for tumors developed by MCF-7 EV cells in the presence of E2 pellets (42.4 ± 4.0 mm^3) ($p<0.071$). Interestingly, unlike the tumor incidence, the mean volumes of O4 tumors were larger in mice treated with E2 pellets (148.3 ± 59.3 mm^3, $p< 0.045$) than in placebo mice. This would suggest that although PCDGF/GP88 overexpressing cells could proliferate in the absence of E2, they still maintained responsiveness to E2 *in vivo*. This finding was compatible with the fact that PCDGF/GP88 overexpressing cells presented similar levels of ER expression than MCF-7 EV cells (20). These data indicate that PCDGF/GP88 overexpression in breast cancer cells increased the tumorigenicity of the cells, and conferred them the ability to form tumors in the absence of estrogen.

Tamoxifen treatment stimulates growth of PCDGF/GP88 overexpressing cells *in vivo*.

Since tamoxifen failed to inhibit O4 cells *in vitro*, its effect on O4 cells tumorigenesis was then examined in mouse xenografts. Female nude mice were implanted with E2 pellets one day prior to inoculating the cells as described in the method section. Ten days later when the tumors were visible, the mice were segregated into groups that received either placebo or tamoxifen pellets and monitored for an additional 45 days as described above. As shown in Fig. 3A, tamoxifen inhibited MCF-7 EV tumor formation (85% inhibition, $p<0.05$), in agreement with the reported inhibitory effect of tamoxifen in ER^+ MCF-7 tumor growth. In contrast, O4 cells formed tumors in mice with or without tamoxifen without any change in tumor incidence (100%). Interestingly, monitoring of tumor volume over the 45 days after tamoxifen implant showed a statistically significant stimulation of tumor growth in the

tamoxifen treated group when compared to placebo control at each time point (Fig. 3*A*). As shown in Fig. 3B, the increase in volume of O4 tumors over the 45 days (V^{45}/V^{0}) was significantly higher in tamoxifen treated mice (6.69 ± 0.43) than in placebo treated mice (4.77 ± 0.70, $p<0.05$). These data demonstrate that PCDGF/GP88 overexpression in breast cancer cells confers estrogen independence and tamoxifen resistance in vivo and that tamoxifen potentiates tumor growth in cells that overexpressed PCDGF/GP88.

It is known that tamoxifen can act both as estrogen receptor agonist and antagonist depending on the tissue types and the distribution of specific estrogen receptor co-activators and co-repressors (23). Since tamoxifen appears to have growth stimulatory activity *in vivo* for PCDGF/GP88 overexpressing cells, we examined whether tamoxifen could be acting as an ER agonist instead of an ER antagonist in these cells. For this purpose, we compared the effect of tamoxifen on ERE-luciferase reporter gene activity in MCF-7 EV and O4 cells in the presence and absence of E2. As shown in Fig. 4, E2 stimulated ERE luciferase activity in both MCF-7 EV and O4 cells by 4.2 and 3.7-fold respectively over the control untreated level. Tamoxifen inhibited E2 mediated stimulation of ERE-luciferase activity by 80% in MCF-7 EV cells and 56% in O4 cells ($p<0.05$). In the absence of E2, tamoxifen stimulated ERE-luciferase activity by 1.6-fold in the PCDGF overexpressing cells ($p<0.05$) whereas it slightly inhibited it in MCF-7 EV cells. These data indicate that tamoxifen maintains its estrogen receptor antagonist effect in PCDGF/GP88 overexpressing cells treated with estrogen. This would suggest that tamoxifen-mediated stimulation of PCDGF/GP88 tumor growth is not simply due to tamoxifen agonistic effect and may involve alternative pathways.

PCDGF/GP88 prevents tamoxifen-induced PARP cleavage

It has been reported that tamoxifen exerts two independent effects on ER^+ breast cancer cells, i.e.: inhibition of estrogen receptor function and activation of apoptosis via down-regulation of bcl-2 (9, 24). Since PCDGF/GP88 confers tamoxifen resistance, experiments were carried out to examine whether PCDGF/GP88 was acting by preventing tamoxifen-mediated apoptosis. Initial studies examined whether PCDGF/GP88 treatment inhibited PARP cleavage induced by tamoxifen in MCF-7 EV cells. Cleavage of PARP is a hallmark of caspases-dependent apoptosis (25). When apoptosis takes place, the 116 kDa intact PARP is cleaved in to 85 kDa and 25 kDa fragments (26). The intact and cleaved forms of PARP were then detected by western blot analysis with anti-PARP antibody. As shown in Fig. 5, tamoxifen treatment induced apoptosis of MCF-7 EV cells, as seen by the increase of cleaved PARP form (85 kDa) when compared to control untreated cells. PARP cleavage induced by tamoxifen in MCF-7 EV cells was inhibited by addition of PCDGF/GP88 (80% inhibition, $p<0.05$), similarly to E2 taken as a positive control.

PCDGF/GP88 inhibits apoptosis by preventing tamoxifen-induced bcl-2 down regulation.

Apoptosis is controlled by the ratio of apoptotic and antiapoptotic factors (27), particularly bcl-2, bcl-x_L and bax (28). Previous reports have suggested that bcl-2 expression was down regulated by tamoxifen treatment leading to activation of apoptosis in MCF-7 cells (9) and in tissues from patients treated with tamoxifen (8). Changes of bcl-2 alter the bax:bcl-2 ratio and affect susceptibility to apoptosis. Therefore, the status of bcl-2 expression in MCF-7 EV and O4 cells treated with tamoxifen was examined. As shown in Fig. 6, tamoxifen induced the down-regulation of bcl-2 transcript in MCF-7 EV cells in a dose dependent manner. In

contrast, tamoxifen failed to down regulate bcl-2 in O4 cells even at doses that completely down regulated bcl-2 expression in MCF-7 EV cells. Interestingly, bax or bcl-x_L expression in MCF-7EV cells or O4 cells remained unchanged at all tamoxifen doses tested.

These data suggest that prevention of tamoxifen-induced apoptosis and bcl-2 down regulation by PCDGF/GP88 is one pathway leading of tamoxifen resistance in breast cancer cells that overexpress this growth factor.

Tamoxifen treatment stimulates angiogenesis in PCDGF/GP88-overexpressing cells.

Our *in vivo* studies have shown that the tumor formation of O4 cells in mice treated with E2 and tamoxifen was increased when compared to mice treated with E2 only. The V^{45}/V^{0} of O4 cells tumors was 1.4-fold higher in mice treated with E2 and tamoxifen than in mice treated with E2 only. This indicates that O4 cells not only failed to be inhibited by tamoxifen, but also were growth stimulated by tamoxifen *in vivo*. Since tamoxifen did not appear to have a direct growth stimulatory effect on PCDGF/GP88 overexpressing cell proliferation *in vitro* (Fig. 1), we hypothesized that the stimulation of tumor growth in nude mice could be due to the *in vivo* recruitment of other growth stimulatory pathways that would provide a growth advantage to the developing tumors. One such possibility was to examine the expression of angiogenic factors in PCDGF/GP88 expressing cells. An angiogenic factor microarray identified VEGFs and angiopoietin-1 and angiopoietin-2 as being expressed by both O4 and MCF-7 EV cells (data not shown). RT-PCR was then used to compare the expression levels of these angiogenic factors *in vivo* in MCF-7 EV and O4 cells tumors obtained from mice treated with E2 alone or with tamoxifen, (Fig. 7). Since tamoxifen induced tumor regression in MCF-7 EV cells in our experiments, MCF-7 EV tumors from tamoxifen treated mice were not included in these comparative studies. The primer sets selected for VEGF permitted to detect five VEGF transcripts: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and

$VEGF_{206}$. Only $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ were detectable in these tumors. As shown in Fig. 7, O4 tumors expressed much higher levels of VEGFs and angiopoietin-1 than MCF-7 EV tumors. Densitometric analysis indicated an 11-fold stimulation of angiopoietin-1 and an 8 to 12-fold stimulation of VEGFs transcripts in O4 tumors. Angiopoietin-2 level remained unchanged. These data indicate that PCDGF/GP88 overexpression strongly upregulates angiopoietin-1 and VEGFs in breast cancer cells. The expression of VEGFs and angiopoietin-1 in O4 tumors from tamoxifen treated mice was 3.2-fold and 1.3-fold higher, respectively than in O4 tumors from placebo treated mice. Tamoxifen treatment of O4 tumors further stimulated the expression of VEGFs (2.5 to 4.8-fold, depending on the transcripts) and angiopoietin-1 (1.3-fold) when compared to O4 tumors treated with E2 only. These data indicate that PCDGF/GP88 stimulates the expression of angiogenic factors *in vivo* and that tamoxifen potentiates PCDGF/GP88 effect.

Discussion

The present study demonstrates that PCDGF/GP88 overexpression confers estrogen independence for growth and tamoxifen resistance to the human breast cancer MCF-7 cells. This was not only shown by overexpressing PCDGF/GP88 in MCF-7 cells but also by direct treatment of MCF-7 cells with PCDGF/GP88. In addition, it was demonstrated that tamoxifen resistant MCF-7 cells, selected by culturing the cells in the continuous presence of the anti-estrogen, expressed higher level of PCDGF/GP88 than tamoxifen sensitive cells (Tangkeangsirisin and Serrero, unpublished results), thereby suggesting a direct relationship between PCDGF/GP88 overexpression and tamoxifen resistance. Estrogen-independent growth of O4 cells was observed *in vitro* as well as *in vivo*. Notably, PCDGF/GP88 overexpressing O4 cells formed tumors in ovariectomized mice even without E2 supplementation, unlike MCF-7 EV cells. The tumor incidence and the tumor volume for O4 tumors in the absence or presence of E2 were significantly higher than for MCF-7 EV cells in the presence of E2. This indicates that the overexpression of PCDGF/GP88 is important for tumorigenesis of ER^+ breast cancer cells. It has been previously reported that the inhibition of PCDGF/GP88 expression in the ER^- MDA-MB-468 cells by antisense PCDGF/GP88 cDNA transfection prevented tumor formation in nude mice (21). The present data provide evidence that PCDGF/GP88 overexpression is also important for the tumorigenesis of ER^+ breast cancer cells. This conclusion is in agreement with our pathological studies reporting high PCDGF/GP88 staining in ER^+ as well as ER^- invasive ductal carcinomas (22). In addition, we have found that O4 cells maintained unchanged levels of functional estrogen receptor expression as shown by the comparable activation of ERE-luciferase reporter gene construct by E2 in O4 cells and in MCF-7 EV cells. These data and the estrogen-independent growth of O4 cells *in vitro* and *in vivo* shown here support the hypothesis that the effect of

PCDGF/GP88 in promoting tumor growth is due to a direct autocrine effect of the growth factor. Although the PCDGF/GP88 overexpressing cells proliferate in estrogen-depleted conditions, they retained growth responsiveness to E2 *in vitro* and particularly *in vivo*. The mean O4 tumor volume was significantly increased in mice treated with E2 pellets, compared to placebo pellets. This would suggest that PCDGF/GP88 does not mediate all of E2 growth promoting effects and that E2 and PCDGF/GP88 signaling pathways have additive effects.

In addition to rendering the growth of breast cancer cells estrogen-independent, overexpression of PCDGF/GP88 led to tamoxifen resistance *in vitro* and *in vivo*. Resistance to tamoxifen in PCDGF/GP88 overexpressing cells was observed *in vitro* by thymidine incorporation and long-term proliferation assays. Overexpression of several growth factors or growth factor receptors has been found to be associated with tamoxifen resistance, especially in MCF-7 cells (5). Her-2 receptor overexpression promotes tamoxifen resistance in MCF-7 (29). However, in this case, the resistance corresponded to a decreased ability of tamoxifen to inhibit tumor growth *in vivo* rather than a complete loss of tamoxifen response. Blockade of MAPK in these cells restored tamoxifen sensitivity (30) suggesting that activation of MAPK was involved in the Her-2 mediated tamoxifen resistance. It has been suggested that cross talk between MAPK and estrogen receptor enhanced ligand-independent ER activation resulting in antiestrogen resistance (31, 32). In contrast, other studies have shown that MAPK activation was not sufficient to confer tamoxifen resistance (33). Recently, another signaling pathway, phosphatidylinositol 3-kinase (PI3K), was also reported to activate ERα in MCF-7 cells (34) and to protect cells from apoptosis (35). Our previous study in MCF-7 cells has shown that PCDGF/GP88 activates MAPK and PI3K signaling pathways (20) and (Chen, Dai and Serrero, submitted for publication). Therefore, PCDGF/GP88 overexpression could contribute to tamoxifen resistance by activating either one or both of these pathways.

Our *in vivo* studies show that the PCDGF/GP88 overexpressing cells not only failed to be inhibited by tamoxifen treatment but in fact formed larger tumors in mice treated with tamoxifen. Growth stimulation by tamoxifen has been previously reported for breast cancer cells transfected with FGF-1 or FGF-4 (36, 37) although the mechanism leading to tamoxifen growth stimulation for FGF-4 overexpressing cells remains unclear. The data presented here offer evidence for two possible pathways by which PCDGF/GP88 expression leads to tamoxifen resistance i.e. inhibition of tamoxifen-induced apoptosis and stimulation of angiogenic factors expression.

It has been reported that the inhibitory effect of tamoxifen on the growth of estrogen-dependent cells involves induction of apoptosis (7, 9, 38). The balance between proapoptotic and antiapoptotic factors determines apoptosis (39). Her-2 overexpression in MCF-7 cells suppresses tamoxifen-induced apoptosis by upregulating bcl-2 and bcl-x_L proteins (40). Moreover, it has been shown that the down regulation of bcl-2 is sufficient to induce apoptosis (9). We demonstrate here that PCDGF/GP88 overexpression prevented bcl-2 downregulation induced by tamoxifen resulting in inhibition of caspase-dependent apoptosis. Tamoxifen had a reduced ability to induce PARP cleavage in O4 cells. In addition, PCDGF/GP88 added exogenously prevented PARP cleavage induced by tamoxifen in MCF-7 EV cells, indicating that it directly prevented tamoxifen-induced apoptosis. The anti-death properties of progranulin (PCDGF/GP88) have been reported recently for the SW13 tumors (41). In this case, it was shown that overexpression of progranulin prevented anoikis of serum starved SW13 cells.

We show here that in breast cancer cells, the overexpression of PCDGF/GP88 renders the cells resistant to cytotoxic therapy. Based on this property, it is postulated that PCDGF/GP88 will have the same effect in the many types of tumors where it is overexpressed (18).

As noted above, only *in vivo* assays in nude mice clearly demonstrated that the tumor growth of PCDGF/GP88 overexpressing cells was increased by tamoxifen treatment. The *in vitro* experiment did not show any increased proliferation of tamoxifen-treated PCDGF/GP88-overexpressing cells. This would suggest that, in addition to acting via a direct autocrine stimulation of breast cancer cells growth, PCDGF/GP88 may also promote tumor growth *in vivo* by indirectly acting on the stromal components found in the environment of the tumor, such as the endothelial cells. One likely mechanism would be by stimulating angiogenesis. Angiogenesis is associated with tumor progression. Continuous tumor growth requires new blood vessels to supply nutrients. The stimulation of angiogenic factors will promote tumor growth, as new tumor vessels will be recruited to the tumor site. Various angiogenic factors have been known to be involved in this process. Among them, VEGF is a major inducer of tumor angiogenesis in breast cancer (42) and is essential for initial *in vivo* growth of human breast carcinoma cells (43). We show here an important upregulation of VEGF and angiopoietin-1 in cells overexpressing PCDGF/GP88 compared to MCF-7 EV cells. This would suggest that PCDGF/GP88 overexpression leads to stimulation of angiogenesis *in vivo*. In support of PCDGF action in angiogenesis, PCDGF/progranulin was also found to potentiate proliferation and promote the formation of tube-like structure in human dermal microvascular endothelial cells (44). No difference in angiopoietin-2 expression could be observed between MCF-7 EV and O4 cells. This is different from MCF-7 cells overexpressing Her-2 in which upregulation of angiopoietin-2 was reported (45).

The role of angiopoietin-1 and angiopoietin-2 remain unclear (46-49). Angiopoeitin-1 has been shown to act as an angiogenic promoter in embryonic angiogenesis, although its role in tumor neovascularization remains unclear (49).

Interestingly, our studies indicate that the levels of VEGF and angiopoietin-1 in O4 tumors were further stimulated by tamoxifen treatment. Although the tamoxifen treated MCF-7 tumors were not examined here, several published reports have demonstrated that tamoxifen inhibited VEGF expression stimulated by E2 in MCF-7 cells (50, 51). It is possible that the upregulation of angiogenic factor expression, particularly VEGF, may be due to the overexpression of bcl-2 observed in PCDGF/GP88 overexpressing cells. Such a relationship between bcl-2 and VEGF expression has recently been reported in human melanoma via stimulation of VEGF mRNA stability and promoter-activation (52). Several cytokines and growth factors such as TNF-α, TGF-β, EGF, IGF-1 have been reported to stimulate the expression of angiogenic factors in several cell types (53). Taken together, these data would suggest that tamoxifen promotes tumor growth in vivo by cooperating with PCDGF/GP88 in stimulating angiogenesis via VEGF.

We have shown previously that inhibition of PCDGF/GP88 expression by antisense transfection or action by treatment with anti-PCDGF/GP88 neutralizing antibodies resulted in inhibition of proliferation *in vivo* and *in vitro* (18). Pathological studies of PCDGF/GP88 expression in paraffin embedded biopsies have shown that 80% of invasive ductal carcinoma (IDCs) stained positive for PCDGF/GP88 with a very high expression (3+) in 60% of IDCs in contrast to normal mammary epithelium and benign tumors. In IDCs, PCDGF/GP88 expression correlated well with prognostic markers such as tumor grade, p53 expression and high Ki67 index (22). These data and the fact that PCDGF/GP88 overexpression in breast

tumors results treated with tamoxifen results in larger tumors in nude mice would suggest that tamoxifen treatment might have adverse clinical consequences for patients bearing PCDGF/GP88 overexpressing breast tumors. In fact, tamoxifen stimulated phenotype has been observed in 6.6 % of patients with ER positive tumors (54). Whether PCDGF/GP88 overexpression is only seen in these cases, or is also implicated in a larger pool of clinical anti-estrogen resistance needs to be investigated.

In summary, the study presented here demonstrates the role of PCDGF/GP88 in the tumorigenesis of ER positive breast cancer cells. PCDGF/GP88 provides growth and survival advantage by acting as a mitogen for breast cancer cells, inhibiting tamoxifen induced apoptosis and promoting tumor angiogenesis *in vivo*. In addition, PCDGF/GP88 overexpression alters cell growth response to estrogen and to tamoxifen.

Figure Legends

Fig. 1. Effect of E2 and tamoxifen on the proliferation of O4 and MCF-7 EV cells.

A: Effect of tamoxifen on DNA synthesis. MCF-7 EV (●),O4 cells (▼) or O7 cells (■) (4 x 10^4 cells/well) were incubated in the presence of 1 nM 17β-estradiol (E2) alone or in combination with increasing doses of tamoxifen. DNA synthesis was measured by thymidine incorporation, as described in the method section. Data are represented as percent of control corresponding to DNA synthesis of cells treated with E2 only. Experiments were performed in quadruplicates.

B: Long-term growth of O4 cells in estrogen–depleted medium. PCDGF/GP88 overexpressing O4 cells were cultivated in phenol red-free DMEM/F12 containing 5% charcoal-stripped FBS supplemented with vehicle (●), with 1 nM E2 alone (▼) or in combination with 1 μM tamoxifen (■). Cell number was counted using hemocytometer. Experiments were performed in triplicates.

Fig. 2. *In vivo* tumor growth of MCF-7 EV and O4 in ovariectomized nude mice in the absence or presence of estradiol.

MCF-7 EV and O4 cells were injected s.c. into two sites into ovariectomized athymic nude mice that had received E2 pellets (60-day release) or placebo pellets. Mice were monitored daily for tumor appearance. Tumor dimensions were measured using a caliper. Tumor volume was determined using the formula provided in the material and method section. Data provided correspond to the tumor volume at 45 days for individual tumors.

Fig. 3: Effect of tamoxifen on the growth of O4 cells in nude mice.

Twenty female nude mice were implanted with E2 pellets one day before O4 cells were injected (two sites per mouse) as described in the method section. After 10 days, when the tumors were visible, the mice were separated in two groups that received either placebo pellets or tamoxifen pellet (referred as day 0). Tumor growth was monitored as described above for an additional 45 days. At the end of the experiments, mice were euthanized and the tumors were excised to extract RNA for the RT-PCR described below.

A: Time course of MCF-7 EV and O4 tumor development in the absence (▼ and ●) or presence (∇ and ○) of tamoxifen. B: Growth of O4 tumor expressed as V^{45}/V^{0} for individual tumors measured at day 45 (V^{45}) after tamoxifen or placebo treatment.

The increase in tumor volume (V^{t}/V^{0}) was expressed as the ratio of mean tumor volume determined at each time point (V^{t}) and over the one at day 0 (V^{0}) (time when tamoxifen or placebo pellets were implanted). Values are expressed as mean ± standard error.

* corresponds to $p<0.05$.

Fig. 4: Effect of E2 and tamoxifen on ERE-luciferase reporter gene activity in MCF-7 EV and O4 cells

Cells were cotransfected with pGL2-ERE-luciferase and β-galactosidase reporter gene constructs. E2 (1 nM) and/or tamoxifen (1 μM were added after transfection. Cell lysates were collected after 36 h later to assay luciferase activity. β-galactosidase activity was used to normalize the transfection efficiency. The data for luciferase activities are expressed as fold of activation above the control untreated cells.

Fig. 5. PCDGF/GP88 prevents PARP cleavage and inhibits apoptosis induced by tamoxifen in MCF-7 EV cells.

MCF-7 EV cells were cultivated in estrogen-depleted medium and treated with tamoxifen (Tam), E2 or PCDGF/GP88 as indicated in the method section. Cell lysates were prepared in RIPA buffer containing 6 M urea for the western blot analysis. The level of PARP cleavage was determined by the presence of 85 kDa band (upper panel). Level of α-actin was determined as internal control for equal loading (lower panel). Data are representative of three independent experiments..

Fig. 6: PCDGF/GP88 prevents down regulation of tamoxifen-induced bcl-2 expression.

Bcl-2, Bcl-x_L and Bax mRNA expression were determined by semi-quantitative RT-PCR of RNA extracted from MCF-7 EV and O4 cells cultivated in estrogen-depleted medium and treated for 24 hr with increasing concentrations of tamoxifen (0.5, 1, and 2 μM) as described in the method section. GAPDH mRNA expression was used as internal control for loading. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

Fig. 7: Upregulation of angiogenic factors in O4 tumors from tamoxifen treated mice.

Expression of VEGF, angiopoietin-1 and angiopoietin-2 was examined by RT-PCR using RNA extracted from MCF-7 EV tumors developed in mice treated with E2 (MCF-7 EV), O4 tumors in mice treated with E2 (O4) and O4 tumors in mice treated with E2 and tamoxifen (O4+Tam). GAPDH expression was included as an internal control. Specific sets of primers used to determine the expression of each transcript are described in the method section.

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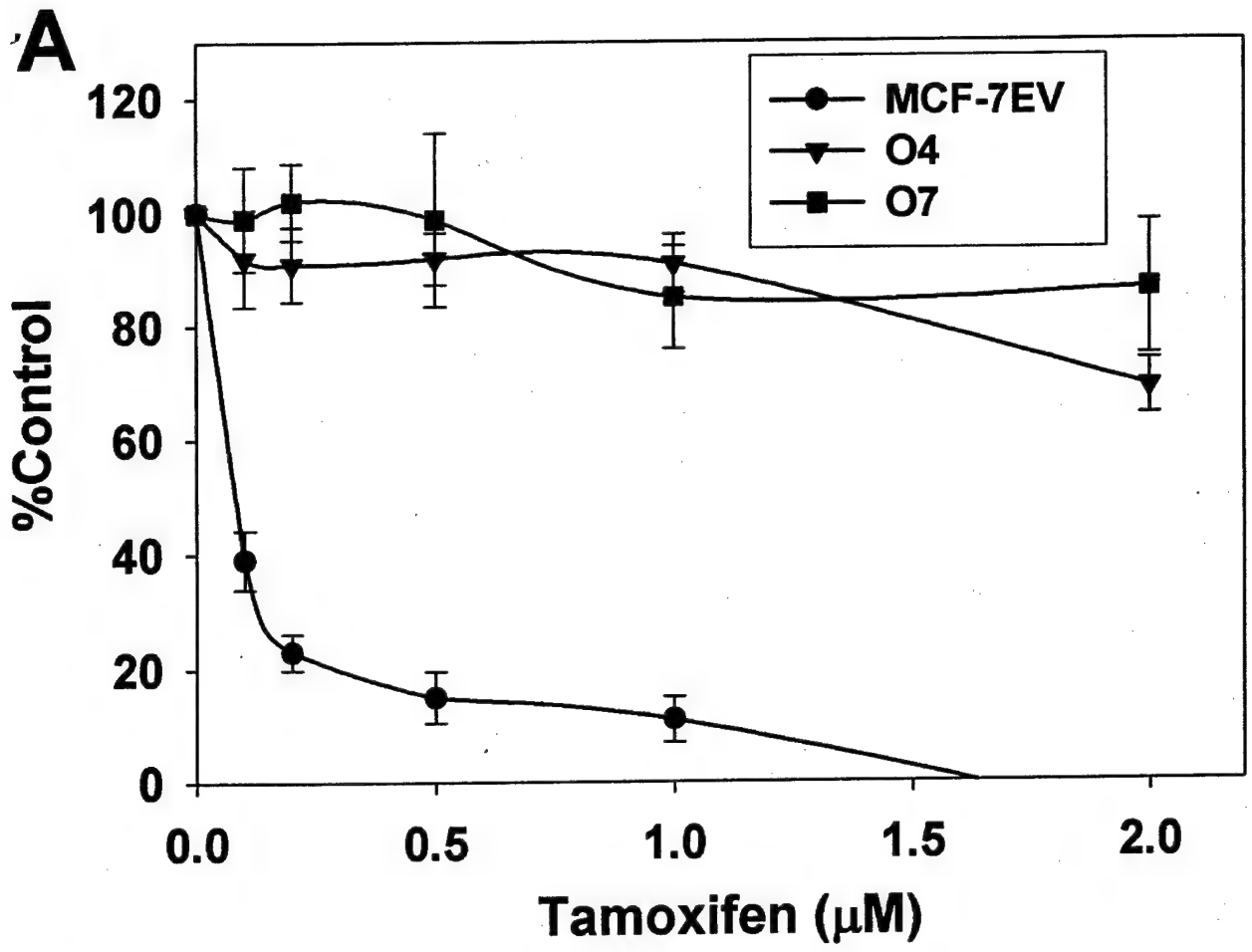
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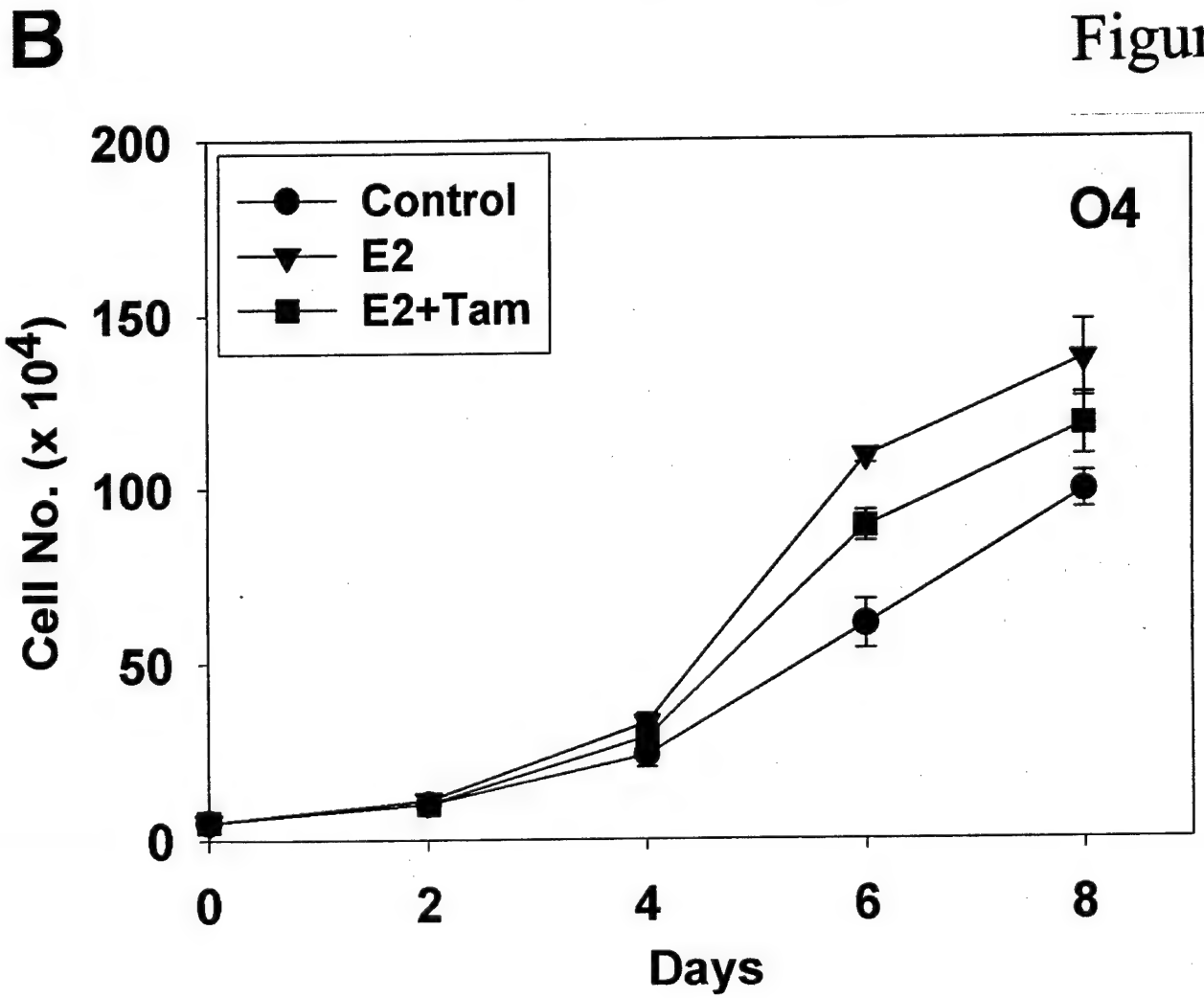
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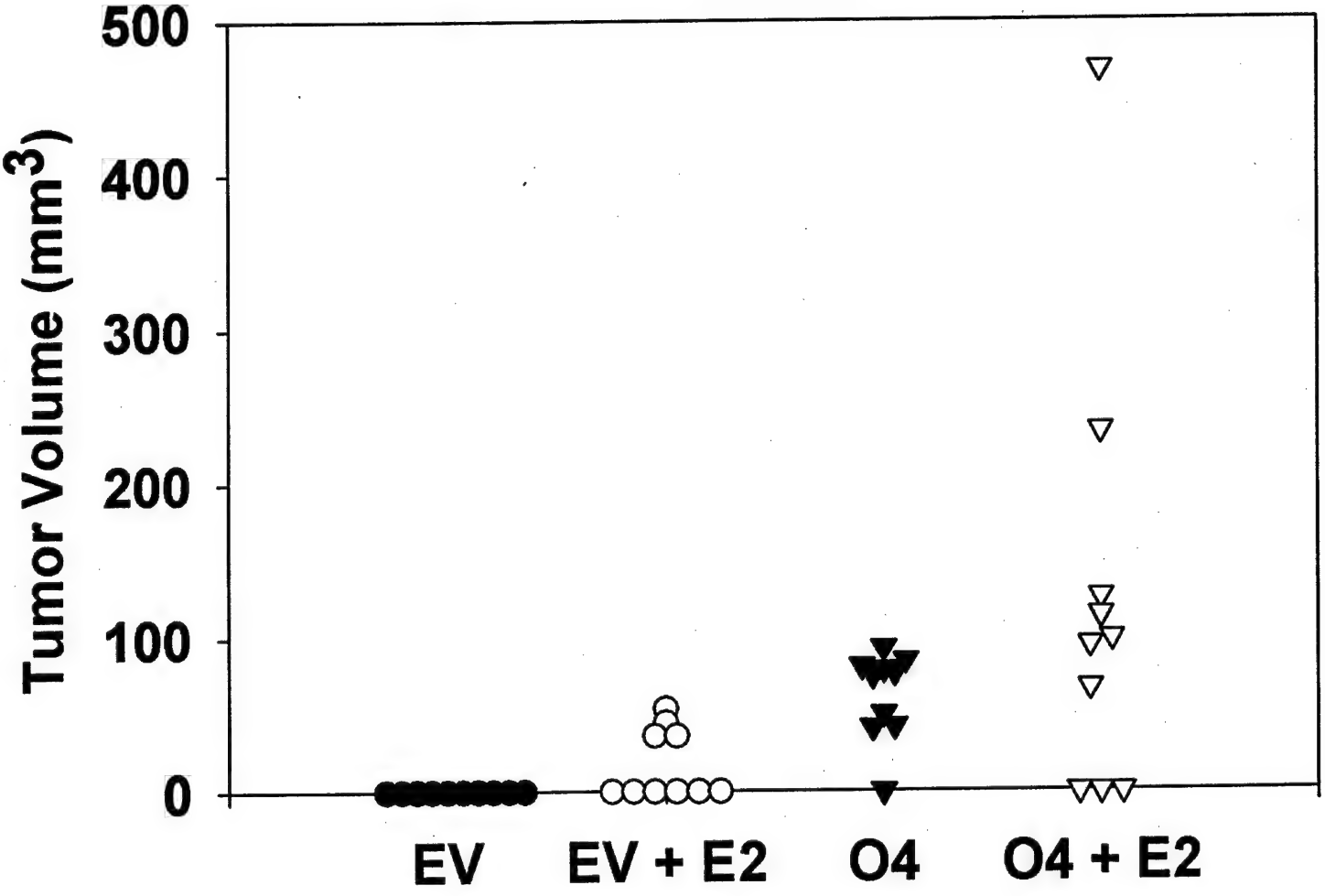
Figure 1

Figure 2



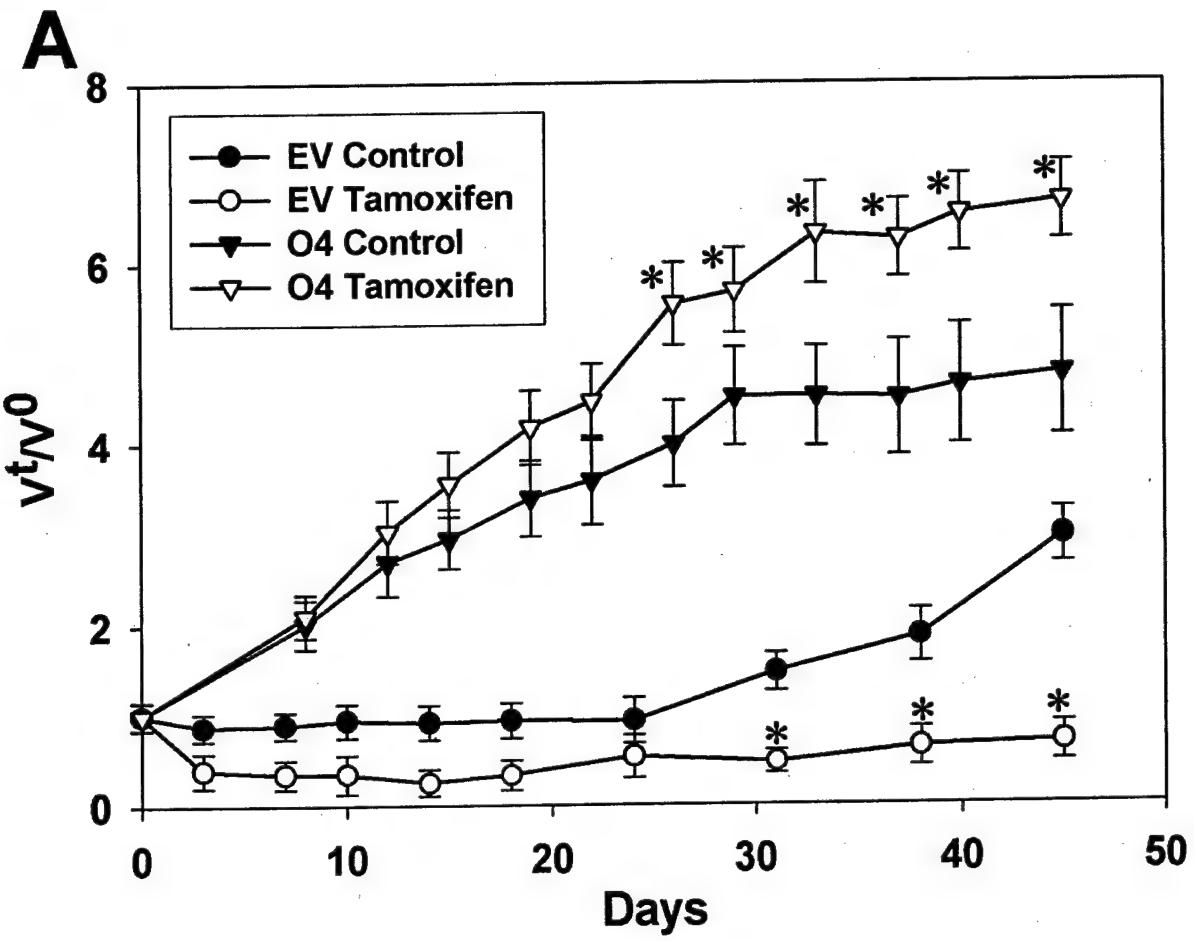

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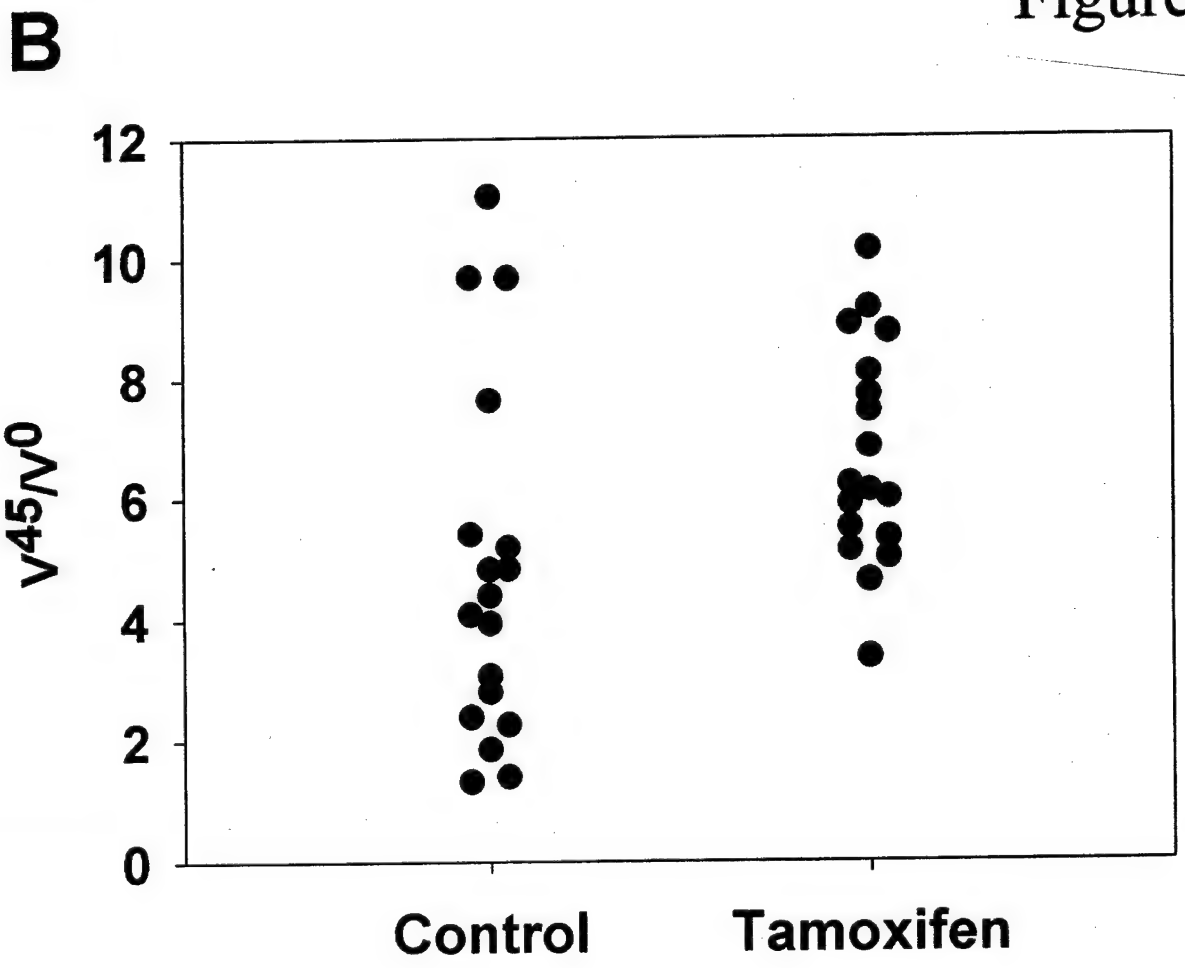
Figure 3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10
12
Control
Tamoxifen

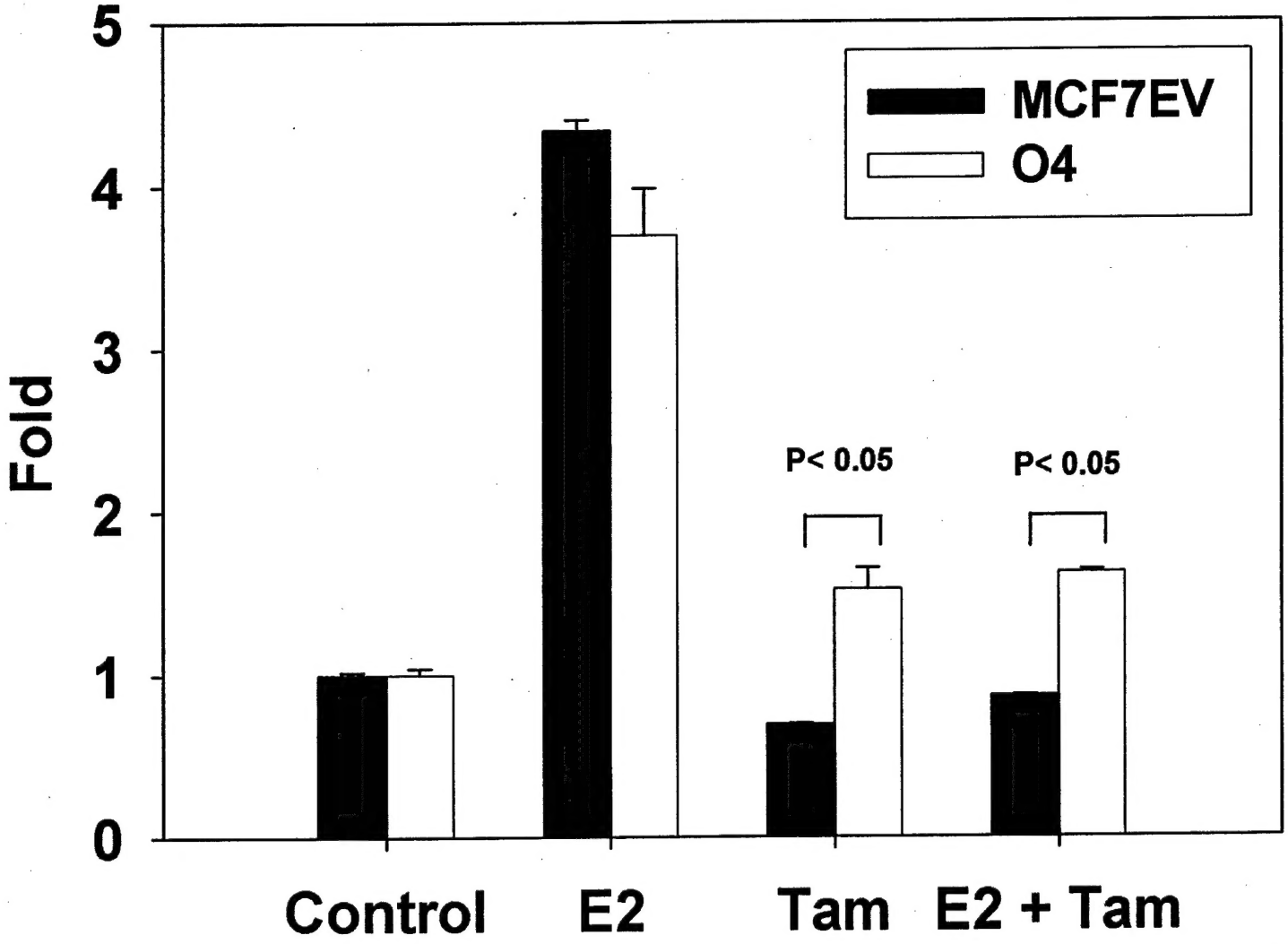

Figure 4

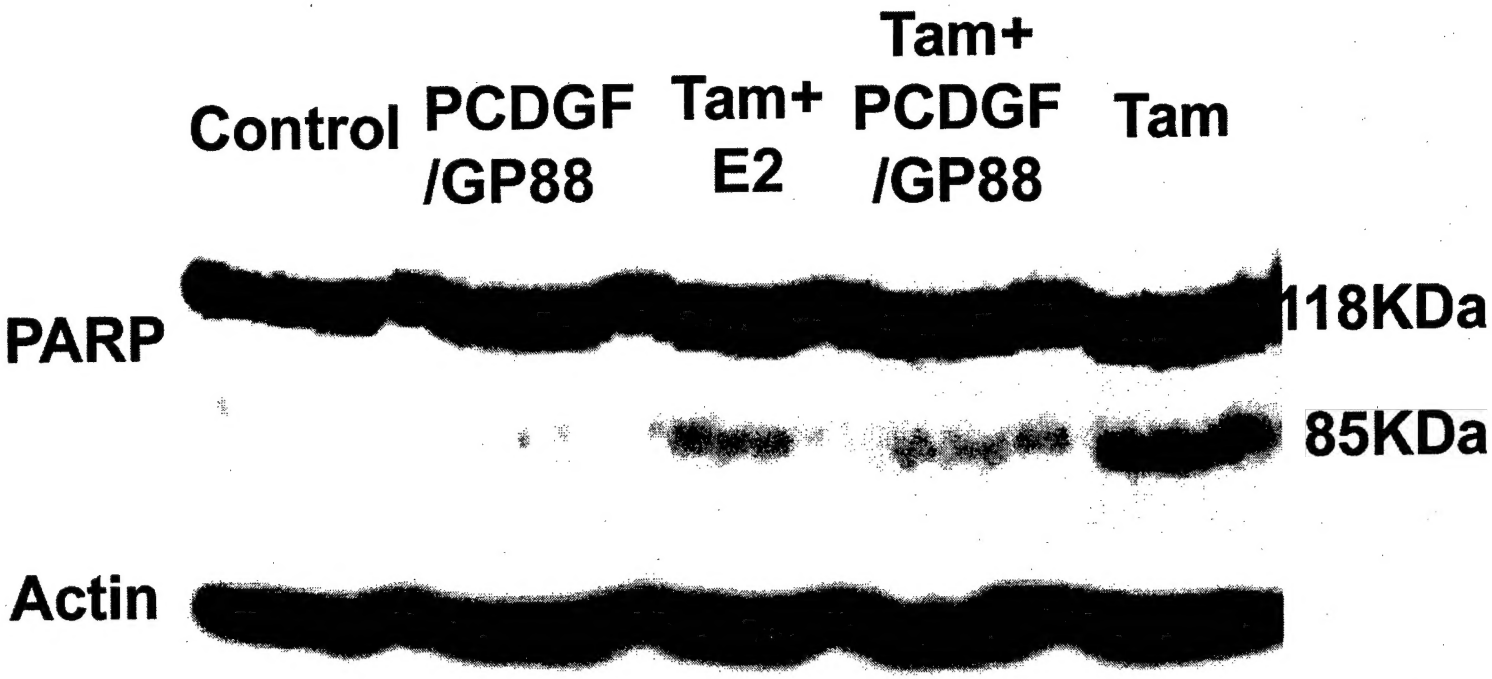
Control
PCDGF /GP88
Tam+ E2
Tam+ PCDGF /GP88
Tam
PARP
118KDa
85KDa
Actin

Figure 5

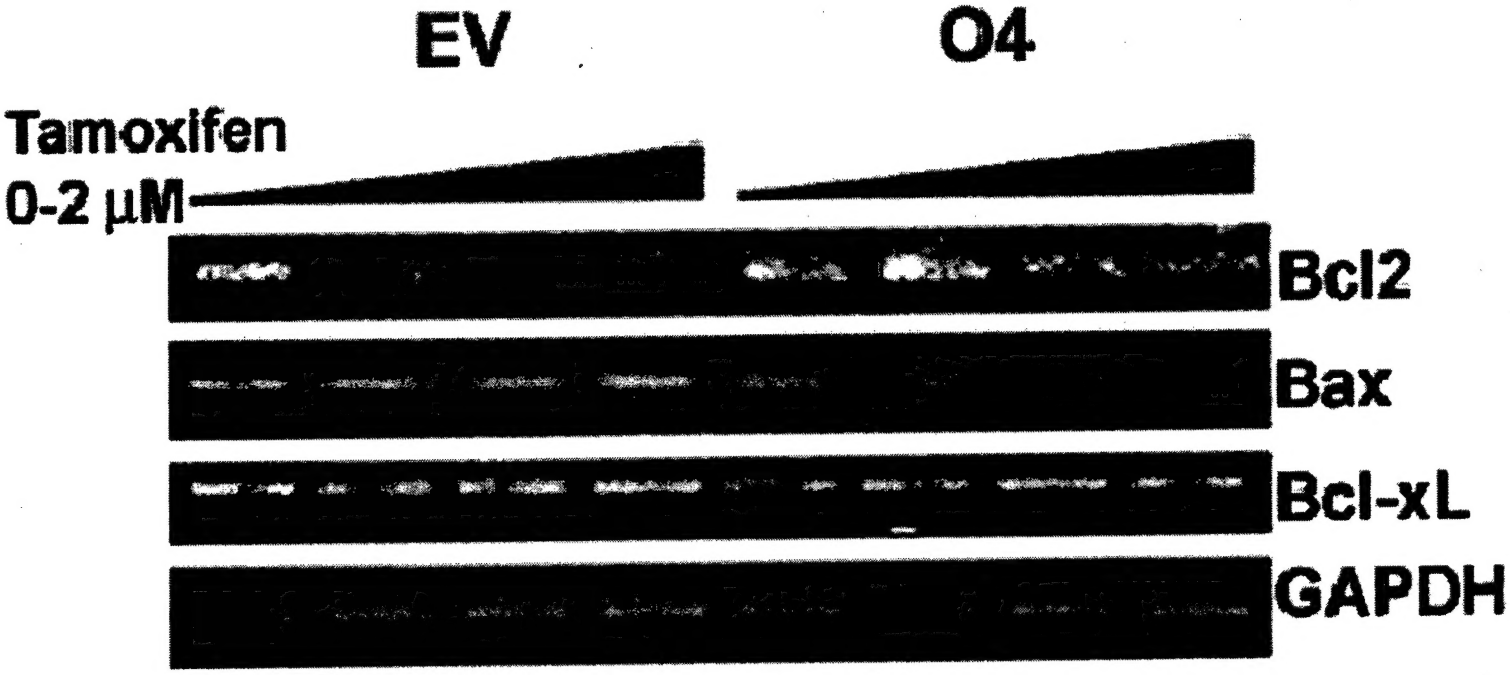

Figure 6

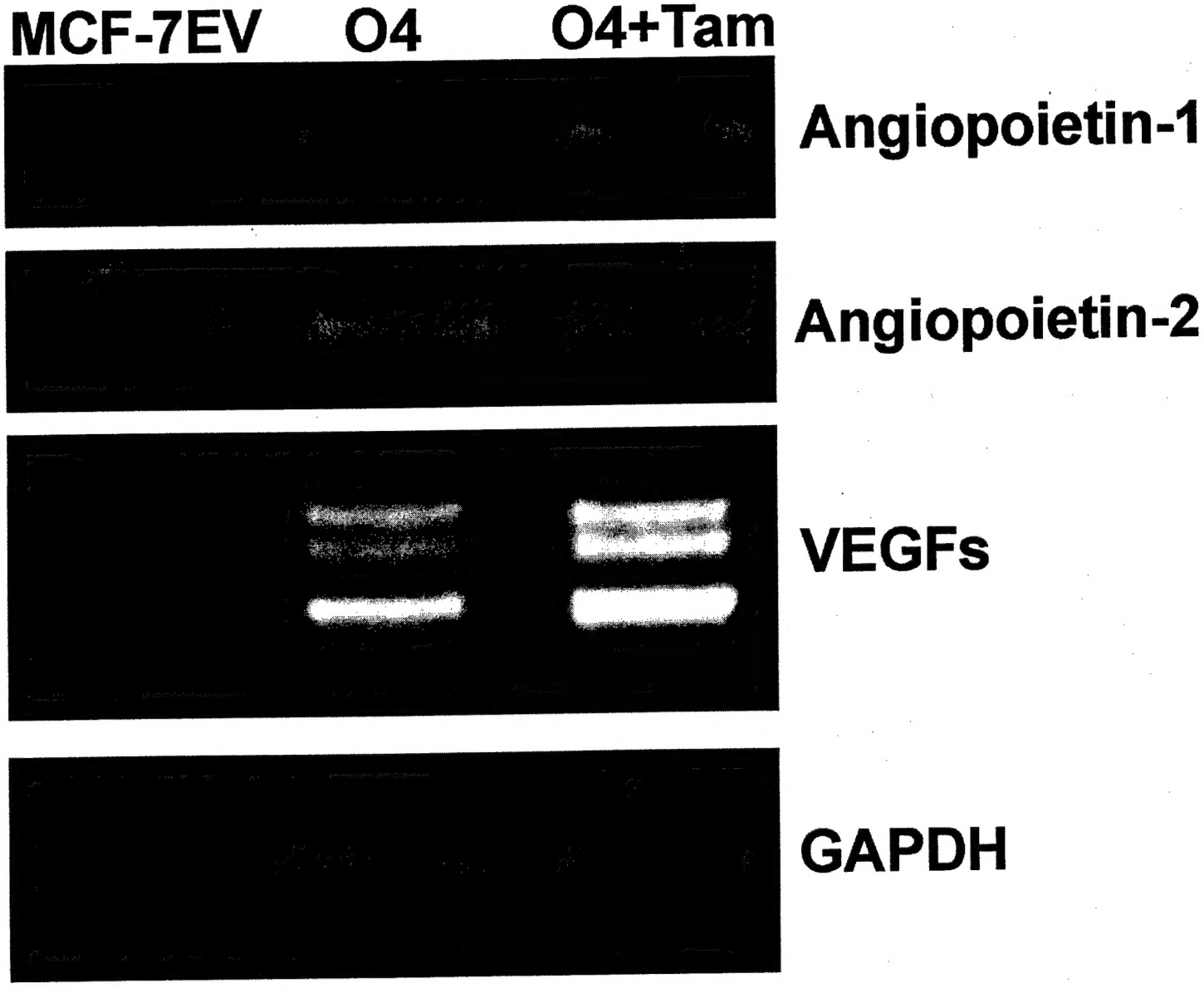

Figure 7